Obituary Notice.

JOHN IRVINE HUNTER,
1898-1924.

The untimely death of Professor John Irvine Hunter has called forth a wonderful wealth of tribute to the memory and to the work of one who, if he had lived, would without doubt have continued to contribute in signal degree to the advancement of biological and medical science in Australia.

Short as the period of his scientific activity had been, he had already achieved results of striking importance, which have served to illuminate some of the obscurer features of muscular activity.

Professor Hunter was a member of the Board of Editors of this Journal, to which also he contributed, whose aim and title so well define the master interests of his own too short scientific career. His was the temper and the mind of the experimentalist. Never content merely to accept on trust, he must prove all things. His genius for research was shown in the devices by which the proofs were to be sought. But, whilst never imposed on by authority, he retained the gift of a singularly open mind, ready, without prejudice, alike to entertain a suggestion or to give full weight to a difficulty or an objection. One could never fail to be impressed by his perfect candour as well as by his sound common sense: both, surely, among the rarer gifts vouchsafed to otherwise able men!

This is not the place appropriate to a detailed memoir of this remarkable and exceptional young man. The salient features of his brief life history have been admirably summarized by his friend, Professor Elliot Smith, like himself a graduate of Sydney, in the columns both of "The Lancet" and of the "British Medical Journal." Here it need only be remarked that his boyhood and his earlier educational career were beset with trials and difficulties both physical and economic. The story of his youth is the story of triumph over obstacles which would have daunted a weaker or a less well-balanced character. His character, so far from being warped, showed in abundant measure the "fruits of the spirit" —love, joy, peace, gentleness, and goodness. Nor did it in any way lack virility. Joyous in activity, physical as well as intellectual, Hunter was a great companion. Something of a hero he was, too, with his fellows, even from undergraduate days. And how characteristic of him that, in the darker days of the war, when he and many of his fellows were perplexed by conflict between the desire to offer themselves for immediate war service, and the ruling of the authorities that they should "carry on," he should go off, without an embarrassing word, to enlist

more or less surreptitiously, only to be sent back to his studies on discovery, after partial training!

Quite early in his undergraduate period he revealed marked gifts for teaching. It was, one surmises, a discovery of himself born of the economic necessity of undertaking the work of coaching. However that may be, as a teacher he grew from strength to strength, becoming first a valuable undergraduate demonstrator, and later, on graduation, a full demonstrator in the Department of Anatomy.

It is surely a matter for congratulation that his University was found ready so far to step out of the rut of ordinary procedure as to promote to the status of Associate-Professor of Anatomy a youth of three and twenty who had graduated only a few months before, and, only a couple of years later, spontaneously to place him in full command of a large department as full Professor. There was never a dissentient voice in regard to these successive appointments, and the confidence of the Senate was amply justified from the first and up to the so lamentably early end.

The same liberality of view was shown by the Senate in its policy of allowing the young Associate-Professor a full year of overseas travel and study at the end of his first year's professorial work, with far-reaching and fruitful result.

But it must not be forgotten that during the year prior to his departure Hunter had already established his position as an original worker by competent and fruitful investigation in several distinct fields of morphological enquiry: in embryology, neurology, and anthropology. And at the same time he had not only carried on the teaching and routine administration of a heavy department during the vacancy in the full chair, but he had inaugurated important changes and extensions of the work of the department. In addition, he had mobilized for research his staff and other volunteer workers in Anatomy, and had roused the enthusiasm and won the affectionate confidence of his students. Collectively all this was an achievement of extraordinary merit, and could only have been accomplished by a rare combination of unflagging industry and natural talent both for organization and for scientific enquiry.

During his fifteen months' absence from Sydney Hunter carried out an extensive and varied programme. He applied himself with the utmost diligence to study along lines, both clinical and academical, collateral to his own subject. He availed himself of the resources of the Royal College of Surgeons Museum under the aegis of Sir Arthur Keith; he busied himself with actual investigation, mainly in the laboratories of the Department of Anatomy in University College, London, under the direction of Professor Elliot Smith, whose influence along with that of Professor J. P. Hill were potent factors in his progressive development; he took some part in the teaching in the same department; and he worked for a short period with Professor Ariëns Kappers in Amsterdam.

He also visited and made himself acquainted with the ideas and methods prevalent in many schools of anatomy both in Great Britain and America.

The entire period of his sojourn overseas was dominated by the idea of future service in teaching and research, and was characterized by eager interest and almost feverish activity.

The fruits were immediately manifest on his return to Sydney. There was no latent period. He had become familiar with the problem concerning the double innervation of skeletal muscle both on its histological and its physiological sides. On his return to Sydney he found Dr. N. D. Royle, with whom he had previously collaborated, deeply engrossed in this question in its practical bearings on spastic conditions in human patients, and at once joined forces with him in an attack on this problem. The brilliant results of this joint enquiry are so well known in scientific circles in Australia that an attempt to set them forth here is superfluous. It will suffice to say that as the work developed it became evident that the workers were on the track of phenomena of far-reaching significance and of vast importance, as well practically as from the point of view of physiological theory.

The surgical world was prompt to recognize the importance of the work from the point of view of surgical practice, and an invitation to Hunter and Royle to deliver the John B. Murphy Oration in New York soon followed their dramatic exposition of their work before the Australasian Medical Congress in Melbourne towards the end of 1923.

The resulting visit to America of Hunter and Royle occasioned there the greatest interest and enthusiasm. Dr. Harvey Cushing has since written in the Boston Medical and Surgical Journal: "The loss which medicine has sustained in the early death of this Second John Hunter is incalculable." Dr. Franklin H. Martin writes in "Surgery, Gynecology, and Obstetrics" that "Professor Hunter and Dr. Royle met with a veritable triumph as they went from city to city, told their story, and enacted their demonstrations. · · · The opinion, I am sure, of those who carefully followed, listened and judged was: 'We have been witness to an epoch-making event in the history of surgery.'"

Hunter decided to return to Sydney by way of England, where he landed in the end of November, 1924. There on several occasions he had the opportunity of giving an account of his work and of setting forth his conclusions, anatomical and physiological. And there also on December 10 came the tragic end to his brief but brilliant life work.

His last contribution to that subject upon which his genius had latterly been concentrated was made in the lecture theatre in Cambridge in the department now presided over by his own old teacher, whose successor in Sydney he had become, and who now mourns the loss of a dearly beloved friend and pupil.

Great as were John Hunter's intellectual gifts, they were not more striking than his graces of character and personality. His utter candour and sincerity were penetrating, and his modesty, not merely of demeanour but of the soul, was itself a witness to real greatness. Such a character, combined with a natural

shrewdness and general soundness of judgment and a wisdom beyond his years, gave confident promise of an influence in the University and in the community at large that must have made him a power for good in his native land, of which he was so loyal and dutiful a son.

Bereaved of his actual presence, his University, and Australia itself, will retain an imperishable memory of this gifted youth, whose short life was lived so fully and earnestly, and withal so joyously, in entire devotion to high intellectual and ethical purposes: a memory which may confidently be looked to to inspire the worshipful youth of Australia to similar lofty and noble ends.

J. T. Wilson.

Anatomy School, New Museums, Cambridge.

HYDROGEN PEROXIDE AND BACTERIAL GROWTH

by

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The action of light in killing or inhibiting the growth of various micro-organisms has long been known. Many authors have observed the directly lethal action of ultraviolet light on bacteria, and there are numerous practical applications of the phenomenon to disinfection. The early experiments were made chiefly with broth cultures, which were inoculated with the bacteria under examination and subsequently exposed to light. The presence or absence of growth on incubation was used as an indication of the sterilizing action of light. Roux (7), in analysing this effect, showed that the media rather than the organisms were primarily affected. Sterile broth exposed to sunlight in the presence of oxygen was found to have become unsuitable for the growth of anthrax bacilli. The phenomenon was further investigated by Dieudonné (2), who related the inhibition to the presence of small amounts of hydrogen peroxide in the exposed media.

More recently the question of the relation of peroxide to the growth of the more delicate organisms, particularly pneumococci, has provoked considerable interest. McLeod and Gordon (5) showed that hydrogen peroxide was produced by several bacterial species in the process of growth, and they have studied the mechanism by which the substance is destroyed in various groups of bacteria. They found that those organisms which show a tendency to die out on sub-culture were in general lacking in catalase activity, and that anaerobes invariably showed no trace of catalase. These observations have been confirmed and extended by Avery and Morgan (1), who found that growth of various anaerobes could be obtained in the presence of air if fresh, unheated potato were present in the medium used. This was ascribed to the presence of peroxidases in the plant tissue. The same method allowed growth of *B. influenzae* without the necessity of haemoglobin being present, and the authors stress the importance of protection of the organisms from small amounts of peroxide produced in the processes of aerobic growth.

The present work is in full accord with these conclusions, although the experimental methods approach the subject from a rather different angle.

It was found that a batch of nutrient agar plates which had been allowed to remain in the light for some time, while still capable of growing staphylococci when heavily inoculated, did not allow growth of isolated organisms. If, however, a broth culture, diluted in saline so as to give discrete colonies, were spread on such a plate, and an adjacent area inoculated heavily with staphylococci, growth of isolated colonies occurred within a range of approximately 1 centimetre from the region of heavy growth. Figure 1 shows a characteristic plate, the control sectors 3-8 representing the typical findings. The present observations were directed toward elucidating the various components of this phenomenon.

1. *The Cause of the Inhibition of Growth.*

It is evident that the plates used either contained a mildly toxic substance capable of inhibiting growth, but not of rapidly killing staphylococci, or lacked some constituent necessary for growth of the organisms. The inhibition occasionally appeared in plates that had been kept for a week or two without more than minimal exposure to light, but was absent in freshly-poured nutrient agar. It could be regularly induced by exposure of the medium in plate or tube to direct sunlight or to the unfiltered rays from a carbon arc. About twenty minutes' exposure to sunlight or to the arc at a distance of 40 centimetres gave the degree of inhibition shown in the various figures. The exposure necessary varied with different batches of agar.

A few experiments were made to determine the region of the spectrum that was effective in inducing the change. An hour's exposure to sunlight through a Wratten filter E (orange transmitting only wave lengths above 5400 A.U.) was ineffective, but with a plate of nutrient agar stained by the addition of 0·08% eosin half an hour's exposure through this filter gave a typical inhibition. Unexposed controls containing eosin showed normal growth. The change induced, therefore, seems to resemble most other photochemical effects in being produced by rays of short wave length only, unless the medium is optically sensitized by a suitable dye.

If media showing this inhibition were heated to 100°C. for thirty minutes they became capable of normal growth, unless the exposure had been extremely prolonged.

From the available literature it seemed highly probable that the change was due to the appearance of hydrogen peroxide produced under the action of light and air. Known amounts of peroxide were therefore added to unexposed plates, and it was found that a degree of inhibition corresponding to that induced by thirty minutes' exposure to sunlight could be obtained when the concentration of peroxide in the medium was approximately 1 : 40,000. Direct evidence of the presence of hydrogen peroxide in exposed plates was also obtained by the

use of a small piece of fresh potato cut from the deeper parts of the tuber, and soaked in a solution of benzidine in glacial acetic acid. Darkening of the surface of the potato occurred only on exposed plates.

The thickness of the layer of agar was found to be important in determining the degree of inhibition produced by a given exposure. A layer over 5 millimetres thick required an extremely long exposure before any marked inhibition occurred, particularly when freshly poured. The plates were exposed as a rule through the under-surface of the agar, and in a thick plate most of the shorter length rays are absorbed before they reach the more oxygenated upper layers, where they can give rise to peroxide. Plates about 2 mm. thick were found convenient in these experiments.

Neutralization of the Peroxide Effect.

In the zone surrounding a heavy surface growth of staphylococci substances diffuse which destroy the effect of the peroxide and allow colonies to develop from isolated organisms. These diffusible substances can be shown to destroy some at least of the peroxide by using the benzidine potato test. This is negative within a zone corresponding to that within which growth occurs. Such peroxide neutralizing substances are also present in broth cultures, and may be demonstrated in an experiment such as the following:

Experiment 1.

A young broth culture of *Staphylococcus aureus* was diluted in saline to 1 : 150 and 1 : 22,500, and single drops of these dilutions were spread alternately on sectors of a plate of nutrient agar (pH 7·6) that had been exposed to sunlight for twenty minutes. An eight-day broth culture of the same organism was centrifugated, and portions of the supernatant fluid was heated to 57°, 75°, and 100° for twenty minutes. A drop of one of these or of normal broth was then added to each sector. Sectors 1 and 2 received that heated to 57°, 3 and 4 received that heated to 75°, 5 and 6 that heated to 100°, and 7 and 8 normal sterile broth. In the centre of the plate a circular area was heavily inoculated from a young agar culture. Figure 1 represents the appearance of the plate after incubation for twenty-four hours. It will be seen that the addition of broth not heated above 57°C. allows growth beyond the region affected by the central area, though even in these sectors the mutually stimulating effect of adjacent growth is evident.

The neutralizing substances in broth lose their activity after being heated to less than 75°C., and may be partly at least of enzyme nature. The power possessed by heated broth cultures from various organisms of allowing growth on peroxide inhibited media was in general parallel to their content in catalase, as determined by the readiness with which oxygen was given off when commercial

3% peroxide was added to them. The organisms examined can be placed in series of diminishing activity according to the catalase content or growth facilitating power of heated broth cultures, as follows:

As catalases: *B. pyocyaneus, Diphtheroid, Staphylococcus aureus, B. coli, B. typhosus. Streptococcus haemolyticus* showed no activity.

As growth-facilitating bodies: *Diphtheroid, B. coli, B. typhosus, Staphylococcus aureus. B. pyocyaneus* and *Streptococcus haemolyticus* showed no facilitating effect with staphylococci.

The presence of various inhibitory substances may be responsible for the irregularities, particularly in the case of *B. pyocyaneus*, which has a well-known inhibitory effect on staphylococcal growth. Evidence will be given later, however, that the two activities are due rather to associated than to identical bodies.

The growth of staphylococci on agar produces readily diffusible substances, which for the most part are destroyed by melting the agar at 100°C. The rate at which the substances diffuse can be seen from the following experiment.

EXPERIMENT 2.

A plate of nutrient agar exposed to sunlight for twenty minutes was kept in the incubator and heavily inoculated with stapylococci over small, separate areas at the times shown. Simultaneously with the last inoculation the rest of the plate was spread with staphylococci diluted in saline to give discrete colonies. After twenty-four hours' incubation growth had occurred within zones around each heavy growth of the radii shown, the rest of the plate appearing sterile.

Time.	Radius of Growth.
42 hours before	3·35 cms.
20 hours before	2·80 cms.
4 hours before	1·56 cms.
0	1·24 cms.

Such a rate of diffusion can only mean that the substances responsible are of moderately small molecular dimensions: no enzyme as ordinarily understood can be involved. Agar containing much of these substances, but free from actual organisms also showed no catalase or peroxidase reactions, and the facilitating effect cannot be accounted for by assuming the activity of such bodies.

In addition to this thermolabile effect, growth of staphylococci on certain batches of nutrient agar for three to five days produces a change in the medium which renders it capable of neutralizing the inhibition of growth by peroxide, even after being heated to 100°C. for several hours. This change in the medium is presumptively taken as indicating the formation by the organisms of a definite substance, which will be referred to as "thermostable X". The following experiment illustrates its characteristic activities.

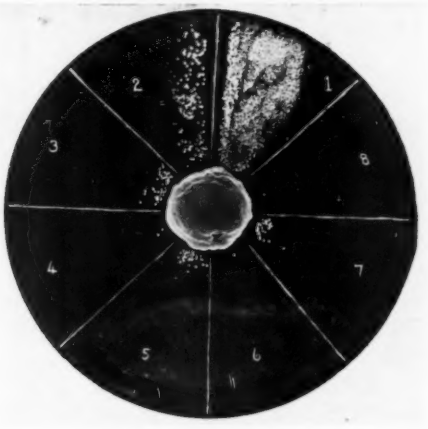

Figure 1.

Light exposed plate spread as described in Experiment 1, to which is added:
Sectors 1 and 2: Broth culture of *staphylococci* heated to 57°C.
Sectors 3 and 4: The same culture heated to 75°C.
Sectors 5 and 6: The same culture heated to 100°C.
Sectors 7 and 8: Normal sterile roth.

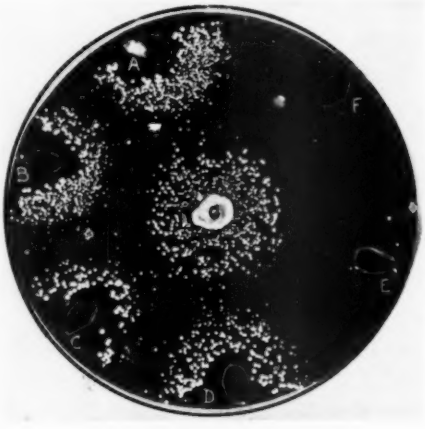

Figure 2.

Light exposed plate spread uniformly with a weak emulsion of *Staphylococci*.

At A. "Vaccinated" agar containing X replacing a small oval of the medium.
B. The same heated and aerated for one hour at 100°C.
C. The same heated and aerated for two hours at 100°C.
D. The same heated without aeration for two hours.
E. Agar "vaccinated" by growth of *B. paratyphosus A.*
F. Agar "vaccinated" by growth of *B. typhosus.*
G. Central growth of *Staphylococci.*

Figure 3.

Plate containing 0.018 potassium cyanide spread as described in Experiment 5.

1. With discrete *Staphylococci.*
2. With *B. typhosus.*

At A: "Vaccinated" agar containing X (staphylococcal).
B. Agar "vaccinated" with *B. paratyphosus B.*
C. Agar "vaccinated" with *B. typhosus.*
D. and F. Growth of *B. typhosus.*
E. Growth of *B. coli.*
Growth of *Staphylococci.*

EXPERIMENT 3.

A number of tubes of nutrient agar on which *Staphylococcus aureus* had been grown for four days were washed clear of growth and melted in the steamer for fifteen minutes. The melted agar was then pooled, and portions treated as follows:

1. Allowed to cool and set aside.
2. Kept at 100°C. with a constant current of filtered air aspirated through the medium. Distilled water was added occasionally to make up for any evaporation.
3. Kept at 100°C. for two hours.

Samples of the aerated medium were taken after one and two hours' heating.

An agar plate was exposed to sunlight for ten minutes to give a fairly weak degree of inhibition. At regular intervals around its periphery small oval pieces of agar were removed with a firm wire ring of suitable shape, and the holes refilled with melted agar treated in one or other of the ways set out above. Agar on which paratyphoid A and typhoid bacilli had been grown was also added in the same way, and a small area at the centre of the plate was heavily spread with *Staphylococcus aureus* culture. The rest of the plate was then spread so as to give discrete colonies of staphylococci. After incubation the plate showed growth as in Figure 2.

The thermostable substance is unaffected by simple heating, but is definitely diminished in activity when actively aerated for two hours at 100°C. Its activity is somewhat less than that of a heavy growth, as judged by the radius over which growth can occur. In all experiments it was found convenient to take the length of this radius as a measure of the amount of thermostable X present. Owing to the variations in the degree of inhibition obtainable in various batches of agar, only measurements derived from the same plate can be properly compared.

The Conditions under which formation of X occurs.

As previously stated, the appearance of thermostable peroxide neutralizing substances is not invariable when staphylococci are grown on nutrient agar, and the whole of the conditions necessary for their appearance have not yet been elucidated. Occasional batches of medium have failed to produce any such effect. The stock medium used in these experiments is made by adding 2% of agar to broth prepared according to Douglas' method from trypsinized veal, and is cleared with egg white. The best results have been with freshly made medium that has undergone only one resterilization and does not easily become inhibitory under the action of light. The reaction is adjusted to pH 7·6.

Of the organisms tested only staphylococci and diphtheroid gave rise to the effect, and *Staphylococcus aureus* was used in all the remaining experiments.

Several days' growth at 37°C. was necessary to obtain an active agar, twenty-four hours' growth being inactive. Anaerobic growth for five days showed no production of X, nor did growth on agar which had received a prolonged exposure to light. The significance of these conditions is not clear, and it seems possible that the thermostable substance is the same as that destroyed by heat, some as yet unidentified physical or chemical condition of the medium allowing it to resist a temperature of 100°.

The Interaction between X and Peroxide.

It was suggested that the increased alkalinity of agar on which staphylococci had been grown might be sufficient to account for the removal of peroxide inhibition. Experiment showed, however, that neutralization of such "vaccinated" agar from its usual pH of about 8·3 to pH 7·0 had no effect on the degree of facilitation produced.

In addition to neutralizing the inhibitory effect of formed peroxide, X will prevent the accumulation of peroxide under the action of light. This can be shown in agar tubes to which a proportion of "vaccinated" agar has been added, both directly by means of the potato benzidine-glacial acetic acid test and indirectly by the ability of the medium to grow discrete staphylococci. The effect depends, however, on the proportion of "vaccinated" agar added and the duration of exposure. In one experiment, for example, a tube containing 30% of agar, on which *Staphylococcus aureus* had been grown for four days, still allowed growth of discrete colonies after five hours' exposure to sunlight, while tubes containing 15% grew well after an hour's exposure, but were rendered inhibitory by five hours' exposure. There is therefore a definite quantitative relation between the amount of X and the amount of peroxide that can be neutralized. Further evidence against the conception that X is a catalyst is given in the following experiment.

Experiment 4.

A series of tubes containing 10% of agar with considerable X activity was exposed to sunlight for four hours. The tubes were then placed in the dark at room temperature, and were inoculated with discrete staphylococci at various intervals up to a fortnight. In all cases a few colonies grew on the thicker part of the slant, but no increase in the extent of growth with lapse of time could be observed.

If the effect of X were a catalytic destruction of peroxide a steady increase in the extent of growth would be observed. It is probable, therefore, that the interaction is a simple reduction of the peroxide, both substances being destroyed in the reaction.

The Effect of Cyanide.

When the action of cyanide was examined on the various reactions that have been described above, it was found that with small concentrations its only

effect was to diminish the range over which light-inhibited agar could be made growth promoting by direct growth of staphylococci or by heated "vaccinated" agar. It seemed quite comparable to a simple addition of hydrogen peroxide. Experiments were therefore carried out to determine whether agar containing cyanide but not peroxide would show a similar phenomenon to that observed with plates exposed to light.

EXPERIMENT 5.

In order to exclude the appearance of peroxide during unavoidable exposure to light, plates were poured containing about 6% of a "vaccinated" agar showing a considerable X activity. To these plates increasing quantities of a potassium cyanide solution were added, and they were then spread in the way shown in figure 3, which is a photograph of the plate containing 0·018% of potassium cyanide. Two-thirds of the plate was inoculated with a suspension of staphylococci to give discrete colonies, the remainder with a similar suspension to typhoid bacilli. A central heavy spreading of staphylococci was added, and around the periphery portions of agar were replaced by "vaccinated" agar on which staphylococci, *B. paratyphosus B.*, and typhoid bacilli had been grown. Small areas were also heavily inoculated with *B. typhosus* and *B. coli*. The results after incubation are tabulated in terms of the radius of growth, in cms., around each source of neutralizing substances in the case of staphylococci, and of the radius over which distinct facilitating effect could be seen in the case of *B. typhosus*.

Percentage of Potassium Cyanide.	0		.006%		.012%		.018%		.024%	
Test Organism.	Staph.	Ty.	Staph.	Ty.	Staph.	Ty.	Staph.	Ty.	Staph.	Ty.
Growth of *Staphylococcus aureus* .	Uniform Growth	Uniform Growth	1·48	Uniform Growth	0·54	Almost Uniform Growth	0·36	0·9	Nil	Nil
Growth of *B. typhosus*			1·70		Nil		Nil	0·7	Nil	* ·46
Growth of *B. coli*			1·15		Nil		Nil	Nil	Nil	Nil
Vaccinated agar *St. aureus* . . .			1·02		0·93		0·65	1·33	0·44	* ·70
Vaccinated agar *B. paratyphosus B.*		NT	Nil	NT	Nil	NT	Nil	NT	Nil	NT
Vaccinated agar *B. typhosus* . .		NT	Nil	NT	Nil	NT	Nil	NT	Nil	NT

NT—Not tested. *These colonies were extremely minute.

It is quite evident that substances produced by growth can annul the inhibitory effect of potassium cyanide as well as that of hydrogen peroxide. The chief difference between the two is that with cyanide the effect of a four-day "vaccinated" agar segment is greater than that of a simultaneous heavy growth, the converse to what has usually been found with peroxide inhibited plates. Peroxide is actually destroyed by X, and it is hard to imagine that the same substance could react directly with the cyanide ion. Much more probably the effect on cyanide inhibition is indirect. In most biological oxidations the paralyzing effect of cyanide is ascribed to its rendering catalytic iron inactive (Warburg) (9). The activity of such iron compounds is usually associated with the "peroxidase" function. Gallagher (3), for instance, regards peroxidase as an oxidation product of a complex aldehyde which acts as an oxygen transporter in the presence of catalytic iron.

In the present example an easy explanation is possible on orthodox lines if we consider that in the presence of air the first step in rendering molecular oxygen available is the formation of a peroxide of some autoxidizable substance which forms hydrogen peroxide in the presence of water. Normally this is removed as fast as it is formed by the peroxidase mechanism of the cell, probably aided by catalase acting on any excess that may appear. If the peroxidase is rendered inactive by the presence of cyanide an accumulation of peroxide takes place in the neighbourhood of the cell, and the condition is quite analagous to what occurs when peroxide is added or produced by the action of light. The toxicity of hydrogen peroxide is, of course, left unexplained. Support of this view is given in the following experiment.

EXPERIMENT 6.

Two series of tubes containing 5 c.cm. of nutrient agar received increasing amounts of potassium cyanide. One of each pair was incubated anaerobically in a McIntosh and Fildes jar for twenty-four hours. These were then inoculated with a dilute enough staphylococcal emulsion to give discrete colonies and reincubated for twenty-four hours in the anaerobic jar. The other series was similarly inoculated and grown aerobically. The degree of growth in the various tubes is shown in the table below.

Cyanide added.	Aerobic Growth.	Anaerobic Growth.
0	+++	++
0·012%	++	++
0·024%	±	++
0·036%	—	++
0·048%	—	++
0·072%	—	+±

The change over the anaerobic conditions necessitates the adoption of a

different metabolic activity in which molecular oxygen plays no part. There is no need for any destruction of peroxides, and the paralysis of peroxidases by cyanide ceases to be of importance. There is a slight degree of inhibition of anaerobic growth in the presence of cyanide, but this can probably be ascribed to interference with other activities that may also be susceptible to cyanide.

DISCUSSION.

The experiments described above have shown that several aerobic organisms, of which *Staphylococcus aureus* has been taken as a type, in the course of growth produce diffusible products which are capable of destroying traces of hydrogen peroxide. By this means they are able to allow growth of isolated organisms that would otherwise fail to develop when spread on a medium containing a little peroxide. This action must be regarded as of teleological significance, for the appearance of traces of peroxide in the environment is almost inseparable from aerobic existence. It is readily produced by the combined action of light and air, and makes its appearance in the autoxidations of living material.

Different bacteria vary greatly in their sensitivity to this agent, as has been shown by McLeod and Gordon (5). Anaerobes are unable to grow in the presence of minute amounts, and amongst the aerobes it is in general the gram-positive organisms that are most susceptible. The order of sensitivity of one series, for instance, was *Streptococcus*, *Diphtheroid*, *Staphylococcus*, *B. typhosus*, *B. pyocyaneus*, *B. paratyphosus B.*, *B. coli*. No satisfactory explanation of the high toxicity of hydrogen peroxide for protoplasm in general seems to have been given. When present in the normal traces produced by autoxidation it is probably rendered immediately available for cell oxidations by a peroxidase mechanism, losing its toxicity in the process. Intra-cellular oxidation systems are invariably highly sensitive to cyanide, and it has been shown above that anaerobic growth is possible in the presence of cyanide at concentrations greater than those completely inhibiting aerobic multiplication. This would mean that with the peroxidase mechanism damaged, growth is possible in the absence of peroxide, but impossible if the conditions for its production (air and autoxidizable substance) are present. But despite such conditions, growth can still take place in the presence of cyanide if products of growth similar to or identical with those shown to be capable of neutralizing peroxide are present.

In discussing the possible explanations of such a phenomenon, it is to be remembered that the current theories of oxidative mechanisms in the cell have been derived chiefly from experiments on tissues of the higher plants. There are bacterial species that derive their energy from very bizarre chemical reactions, and analogies between metabolic processes in bacteria and those in higher forms of protoplasm must be made with caution. It is probable, too, that the differences observed under the various conditions used experimentally are due rather to

quantitative than to qualitative changes. The effect of cyanide on oxidative processes is generally ascribed to inactivation of catalytic iron, but its exact nature is still dubious. Such peroxidase phenomena as the benzidine reaction are resistant to high concentrations of cyanide, although the appearance of the blue colour is proportionately delayed. In any case, the action of cyanide is a quantitative one, as Experiment 5 clearly shows. The radius of growth surrounding a constant source of neutralizing substances (the heated "vaccinated" agar) is inversely related to the concentrations of cyanide used, which suggests that there is some similar relation between cyanide and the substance primarily destroyed by such growth products. If this be hydrogen peroxide, we must assume that the action of increasing amounts of cyanide is to render the organism progressively more sensitive to its toxic effect, probably by progressive weakening of the mechanism for its removal. The diffusible products of staphylococcal growth can remove peroxide as it is formed, and the effect of cyanide is thus minimized as long as any power of respiration remains.

Products of growth do not neutralize the effects of peroxide and cyanide in exactly the same way, as can well be seen in two different types of experiment: (1) In the table under Experiment 5 it will be noticed that the zone of facilitation on cyanide plates is greater with the active "vaccinated" agar than with the living growth of staphylococci, and that the difference is more marked in the higher cyanide concentrations. The converse is the invariable rule when peroxide plates are used. (2) If two plates are spread in sectors with dilute suspensions of various micro-organisms, and a few drops of strong peroxide in one and of cyanide in the other are placed in a hole at the centre, incubation will show characteristic differences. The plate to which peroxide was added will show a regular line at a distance from the centre varying with the organism, on one side of which the surface is sterile, on the other covered with colonies of uniform size, whereas on the cyanide plate there is a broad zone between the sterile region and the periphery covered with colonies of gradually increasing size. In both these experiments the differences are due to the fact that if an organism under any conditions gives rise to a colony on a plate containing peroxide, the colony will cause the continuous destruction of any further peroxide diffusing into its environment. Practically speaking, it will be growing on normal agar, and will produce its normal amount of growth products. With cyanide, however, neutralization is indirect, and the colony can only protect its individuals by destroying peroxide. There is no destruction of the primary toxic agent, and with increasing concentration growth becomes more and more inefficient and less peroxide neutralizing substances are produced.

It must not be forgotten that staphylococci and most of the other easily cultivated pathogens are readily capable of anaerobic growth. If the current views as to the importance of peroxide formation in the first stages of cell respiration are correct, the abundance of catalase and reducing bodies in the substance

of the colonies would hardly allow aerobic growth. It is much more likely that, with the exception of organisms in direct contact with the air, the type of metabolism is anaerobic, as would be the normal mode of existence of these bacteria in their natural environment. Traces of peroxide are so toxic to organisms in the presence of cyanide that it is probable that the actual presence of peroxide in the environment is the stimulus causing the assumption of aerobic metabolism. Otherwise one would expect that the organism would adopt anaerobic activity, toward which cyanide has been shown to be practically innocuous. Aerobic growth may be regarded as an adaptation to deal with unfavourable conditions, until with the growth of the colony reducing substances are produced, which by destruction of any peroxide formed, allow at least a predominant maintenance of anaerobic conditions. Unless this view is adopted it is difficult to understand why the various peroxide-destroying substances are produced in such abundance. If their function is to maintain what may be termed the internal environment of the colony at the particular degree of anaerobiasis that is conducive to the most effective growth of the organism, the facts become readily intelligible. Evidence in favour of this view may be drawn from several sources. McLeod and Gordon (6) have shown that anaerobes may grow well on suitable media in the presence of air, but that minute traces of peroxide will entirely prevent growth. The peroxide may perhaps be a stimulus to a metabolic activity that the organism is incapable of pursuing. The old observation of Kedrowski (4) and others that anaerobes may be grown in the presence of various aerobic organisms, although free access of air is allowed, helps to relate the phenomena observed with anaerobes to those found with staphylococci, and suggests that the products of the aerobic organisms responsible are substances which destroy hydrogen peroxide. If they will allow growth of strict anaerobes it is highly probable that they may permit anaerobic growth of a facultative anaerobe in the presence of air. Direct evidence that such anaerobic growth may occur was obtained by Stephenson and Wheatham (8), who showed that *B. coli* in 1% glucose broth grows anaerobically unless oxygen at a considerably higher tension than that of air is supplied.

Staphylococci, then, are capable of obtaining their energy by various means, of which the strictly aerobic and anaerobic methods are the two extremes of a continuous series. The most readily assumed type lies near the anaerobic end of the series, but in the presence of peroxide either originally present in the environment or produced by autoxidation of bacterial substance, aerobic activity results. Growth and multiplication continues until an accumulation of peroxide-destroying bodies gradually reduces the hydrogen peroxide in the immediate neighbourhood to a low enough level to allow the optimal method of growth.

In the case of an obligate aerobe, such as *B. subtilis*, it is found that products of growth of staphylococci can protect isolated organisms against small concentrations of cyanide, and that homologous products have a similar though less

active effect. Here there can be no assumption of anaerobic metabolism, but the partially poisoned oxidative mechanism can function effectively if the concentration of peroxide can be kept low enough. The peroxide neutralizing bodies in both cases may have an additional function and serve as a thermostable oxygen transporter (somewhat analagous to glutathione), which renders peroxide oxygen available to the cell without the intermediation of the cyanide-sensitive mechanism. No direct evidence of such a function, however, has been obtained, and agar showing high X activity gives no nitroprusside reaction.

SUMMARY.

1. Exposure of nutrient agar plates to light results in the appearance of traces of hydrogen peroxide sufficient to inhibit the growth of isolated staphylococci and other organisms.

2. This inhibition can be neutralized by diffusible products of growth of staphylococci.

3. These diffusible substances, some of which are thermostable, act as reducing agents, destroying any peroxide as it is formed.

4. An inhibition of growth due to potassium cyanide can also be neutralized by these substances.

5. Anaerobic growth of staphylococci is possible in concentrations of cyanide higher than those completely inhibiting aerobic development.

6. It is concluded that the presence of catalase and of the diffusible substances studied in the bacterial colony is an indication of a primitive means of keeping constant the immediate environment, so as to allow the type of metabolism most suited to the organism to be maintained.

I wish to express my obligation to Dr. C. H. Kellaway and Professor W. A. Osborne for criticism of parts of this work, and to the latter for allowing some experiments to be carried out in the Physiological Laboratory, University of Melbourne.

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A NOTE ON THE EFFECT OF DYES ON BACTERIAL GROWTH

by

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In looking over the literature on disinfection and on growth inhibition of bacteria, one is struck by the way in which gram-positive are in general much more sensitive than gram-negative organisms. This is particularly evident in the case of dyes. Eisenberg (4) examined 115 dyestuffs of a wide range of constitution, and found throughout the series that gram-positive organisms were from 3 to 10,000 times as sensitive as representative gram-negative ones *(B. coli* and *B. typhosus)*. The generalization does not hold very strictly, and there is considerable variation, particularly amongst the gram-negative group. The pathogenic gram-negative cocci behave like the gram-positive cocci: *B. pneumoniae*, *V. cholerae*, and the dysentery bacilli are moderately sensitive to most dyes. The spore formers, *B. subtilis* and *B. anthracis*, are the most sensitive of all the aerobic bacteria. These results were obtained by exposing organisms to various concentrations of the dyes in fluid media, usually distilled water, and spreading them out on suitable solid media after the required interval.

Churchmann (3), using similar methods, finds that with certain dyes, of which acid fuchsin is representative, gram-positive spore-bearers of the *subtilis* type are less sensitive than gram-negative organisms. Burke and Skinner (1) are probably right in regarding this as merely indicating that the spores of these organisms are resistant, the vegetative forms being as sensitive as the usual gram-negative bacteria to these dyes. The fact that media dyed with fuchsin show the usual selective inhibition of such gram-positive organisms (Churchmann's "reverse bacteriostasis") is strongly in favour of this interpretation.

The general trend of these results is remarkably parallel to the sensitivity of different bacteria to hydrogen peroxide. In the preceding paper evidence was put forward that seems to strengthen the view of McLeod and Gordon (6) and others that the reactions to peroxide are of fundamental importance to the life of the bacterium. It was therefore of interest to determine if the phenomena observed with peroxide and cyanide could be obtained with various dyes. Acid

fuchsin was chosen on account of the work that has been done with it by Churchmann and others in America.

In the course of the work previously reported it was found that *Staphylococcus aureus* and a diphtheroid bacillus were very sensitive to peroxide, and showed well-marked zones of growth facilitation around a source of neutralizing substances. Amongst the gram-negative organisms strains of *B. pneumoniae* Friedlander, *B. typhosus*, and *B. coli* formed a series showing increasing resistance to the inhibitory effect. The first was almost as sensitive as *Staphylococcus aureus*, *B. typhosus* was rarely inhibited on plates exposed to light, and *B. coli* never. *B. pyocyaneus* was very similar to *B. typhosus* in its reactions.

Figure 1 represents the result of an experiment in which sectors of a nutrient agar plate containing 0·5% of a saturated aqueous solution of acid fuchsin were spread with dilute saline suspensions of these organisms. In the centre a small area was heavily inoculated with staphylococci. The plate was a freshly-poured one and exposed only to weak artificial light. On incubation it will be seen that no inhibition of *B. coli*, *B. typhosus*, or *B. pyocyaneus* has occurred, but *Staphylococcus aureus*, a diphtheroid bacillus, and Friedlander's bacillus have reacted precisely as would have been expected in a plate containing peroxide or cyanide, growth occurring only in a zone surrounding the staphylococcal growth. The fact that Friedlander's bacillus, although gram-negative, reacts in this way both to peroxide and to fuchsin makes it impossible to avoid the conclusion that the two agents are in some way related in their effect on the various organisms. The neutralizing influence here seems to be the same as that discussed with peroxide. Agar containing the thermostable X factor showed diffusion of substances neutralizing the effect of fuchsin on isolated staphylococci. As in all the experiments showing this phenomenon, the results are quantitatively determined; neutralization is only possible below a certain concentration of dye that varies for each organism.

The relationship between these results and those obtained with hydrogen peroxide are capable of several interpretations. The alternatives to be considered are:

1. That fuchsin causes the formation of peroxide in the medium without more than a minimal exposure to light being required.
2. That sufficient peroxide is present in the watery solution of the dye to give its characteristic effect when added to the agar.
3. That the effect of the dye is on the organism by either (a) increasing its production of peroxide; or (b) hindering the removal of normally produced amounts.

No evidence for the first two alternatives has been obtained, though owing to the inadmissibility of colour tests, the first is difficult to refute directly.

Nutrient agar containing a smaller proportion of fuchsin than that necessary to inhibit staphylococcal growth required, in order to become inhibitory, about

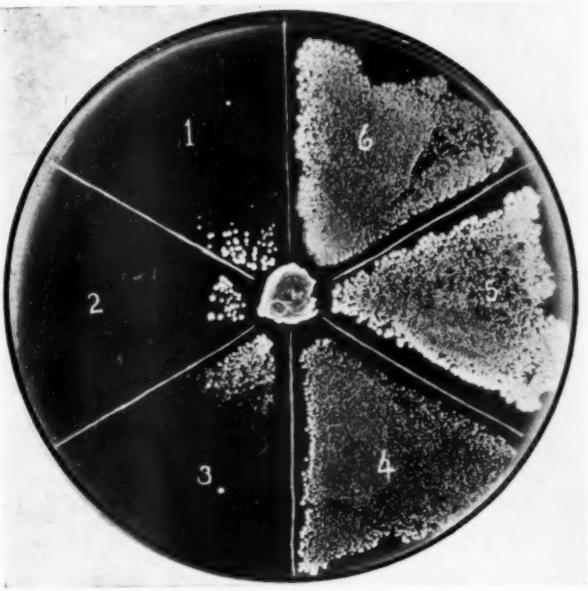

Figure 1.

Plate containing 0·5% saturated aqueus solution of acid fuchsin with central staphylococcal spreading. Sectors spread with suspensions of:

1. *Staphylococcus aureus.*
2. Diphtheroid bacillus.
3. Friedlander's bacillus.
4. *B. typhosus.*
5. *B. typhosus.*
6. *B. pyocaneus.*

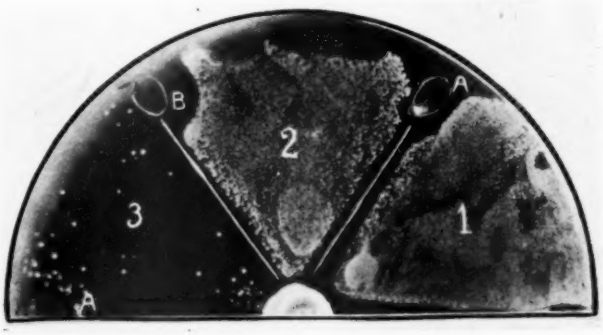

Figure 2.

Plate rendered slightly inhibitory by exposure to light: central staphylococcal spreading. At A and B agar replaced by samples of "vaccinated" agar containing X. Sectors spread $\frac{1}{250}$ dilutions in saline of young broth culture of *B. typhosus:*

1. Unheated.
2. After two minutes' heating to 56°C.
3. After five minutes at 56°C.

the same exposure to light as a control. This inhibition could be destroyed by incubation under anaerobic conditions for two days or by heating to 100° for thirty minutes as readily as that of the control. It seems unlikely, therefore, that the addition of fuchsin makes any great difference in the conditions under which peroxide is produced and retained in the medium.

As regards the second alternative, there is no reason to expect such occurrence of peroxide in the dye solution, and no oxygen could be liberated from it by the usual peroxide decomposing agents.

We are left with the probability that the fuchsin interferes directly with the production or utilization of peroxide by the organism. The presence of products of growth, whose primary function is to destroy peroxide, protects isolated organisms against otherwise inhibitory concentrations of the dye. Like cyanide, fuchsin must act by rendering the organism sensitive to traces of peroxide produced in its own metabolism. This sensitivity is probably due to injury to the mechanism for the normal utilization of peroxide. The effect differs from cyanide in some respects. When an agar plate was spread as in figure 1, and, after drying off, incubated anaerobically in a McIntosh and Fildes jar, the result was very similar to that obtained aerobically, although the radius of growth was a few millimetres more extensive. Under the same conditions a cyanide plate gives uniform growth of staphylococci. The accumulation of peroxide derived from autoxidation during the period before relatively complete anaerobiasis is obtained must be in some way more intimately related to the cell than is the case with cyanide. Under the latter circumstances anaerobiasis permits the diffusion of any peroxide that has accumulated, and in the absence of further production growth occurs. With fuchsin it appears that the peroxide responsible for the inhibition is not free to diffuse, and can only be rendered nugatory by the presence of actual reducing substances in its immediate environment.

Oerskov (7) has recently reported experiments which show that typhoid bacilli and other members of the coli-dysentery group on being heated for a short time at 55°C. may lose their power to grow aerobically on an agar surface while still capable of multiplication under anaerobic conditions. It seemed probable that this failure to grow aerobically might be related to the phenomena encountered with cyanide or fuchsin. As described above, *B. typhosus* in its normal condition grows readily on agar that contains enough peroxide to render it inhibitory for isolated staphylococci. When heated organisms were used the results were somewhat inconstant, but, as will be seen from figure 2, a typical peroxide inhibition could sometimes be obtained. The sectors here were each spread with a drop of 1 : 250 saline dilution of samples taken from a young broth culture of *B. typhosus* before and after two and five minutes' heating respectively in a waterbath at 56°C. The plate had been exposed to light for twenty minutes, and on the half omitted from the figure showed a typical facilitation of staphylococcal growth. At the centre is the usual heavy staphylococcal spreading, and

A and B represent two samples of agar containing thermostable peroxide neutralizing substances. It may be concluded that by a degree of heat which is just sub-lethal typhoid bacilli can be rendered abnormally susceptible to hydrogen peroxide, and that the inhibition so caused can, as in naturally susceptible organisms, be neutralized by certain products of bacterial growth.

It has been shown that the effects on the growth of bacteria of such different noxious agents as heat, acid fuchsin, and potassium cyanide, as well as hydrogen peroxide can be neutralized *to a certain degree* by what are almost certainly the same products of growth in each case. This suggests that in different ways these agents, and probably many others, all affect first one and the same function of the growing organism. This function can only be the power possessed by the organism of dealing effectively with hydrogen peroxide as it is formed. It is this function then that we must regard as the vulnerable point of the organism in relation to its environment.

A bacterial colony may be merely a fortuitous result of continued multiplication, but it seems, too, to furnish a means whereby numerous potentially noxious influences are rendered less effective. An isolated organism may produce diffusible substances as readily as one within a colony, but it cannot retain them within its immediate neighbourhood, and a high concentration of such substances can only occur when numerous adjacent organisms collaborate in their production. The most important peroxide-destroying bodies have been shown to be diffusible, and will therefore reach a high concentration only when a colony is formed. By their presence traces of peroxide in excess of the amount which can be dealt with by the oxidative mechanism of individual organisms are immediately destroyed, and in this way the viability of cells damaged by small changes in the environment is retained. It may be considered as a primitive maintenance of constant internal environment by ensuring that the immediately toxic body in many cases of bacterial injury (hydrogen peroxide) shall not accumulate. In this respect the bacterial colony may almost be regarded as a metazoan individual.

Some aspects of the growth inhibiting action of dyes may be rendered more intelligible by these ideas. The regularity with which gram-positive organisms are more readily inhibited than the common gram-negative ones can be understood if the action of the dye at the limiting dilutions falls mainly on the function of peroxide destruction. The organisms which are most sensitive to peroxide in their normal state may be expected to be most sensitive also to any "pathological" accumulation. The order of sensitivity of the readily cultivated non-sporing pathogenic organisms which are facultative anaerobes is the same for the great majority of dyes as it is for hydrogen peroxide. Thus both the gram-negative organisms which show an unusual sensitivity to peroxide, *B. dysenteriae* Shiga and *V. cholera* (McLeod and Gordon 6) are similarly susceptible to most dyes (Kligler 5) (Eisenberg 4), and it has been noted above that Friedlander's

bacillus, although gram-negative, is sensitive to both peroxide and fuchsin. In the case of spore-formers, strict aerobes and anaerobes, the simple parallelism may not apply. I have been unable to find references to the action of dyes upon anaerobes, but with *B. subtilis* dyes are relatively much more powerful inhibitors than is hydrogen peroxide. This organism is a strict aerobe and a spore-bearer, and hypothetical explanations of its behaviour are not difficult to conceive. They would, however, be lacking in value till a wider comparative study of these groups of bacteria had been made.

The neutralizing effect of products of growth on various dyes has been noted previously, particularly by Churchman (2), who discusses it under the term "communal activity of bacteria". He found that from a strain of *B. coli*, apparently quite resistant to gentian violet *en masse*, no growth of isolated organisms could be obtained on agar containing the dye, nor could bacilli isolated by a single cell technique be induced to grow in gentian violet broth. When about thirty organisms were used as the inoculum growth occurred. One of his figures of strokes of dilute coli emulsions spread on divided agar plates shows growth on the gentian violet side of the plate, only within a zone extending for about 1 centimetre from the growth of normal agar. This appearance must be essentially of the same nature as those described above with fuchsin, and due to diffusion from the normal growth of similar substances.

Such a description of the mode of action of many dyes at their limiting inhibitory concentration is in no sense a complete explanation of their effect. The description is a purely functional one, and the way in which numerous substances of very different chemical nature can affect a single function is quite obscure.

It has been noted already that there is a difference in the way cyanide and acid fuchsin work, in that the inhibition of the first is much more readily overcome by simple anaerobiasis than is the other. With other agents still further variations may be expected, and in isolated instances the dye may first affect quite a different function of the organism, e.g., in experiments with *B. typhosus* and a dye to which it is very highly resistant (gentian violet) there was a definite relative inhibition in the neighourhood of a source of diffusing growth products. Such variations in particular instances probably arise from interactions between specific constituents of the organism and the dye. The general rule is not thereby invalidated that the great majority of non-specific lethal agents affect first the peroxide utilizing function, and that the degree of injury to this that can be withstood is in general parallel to the resistance of the organisms to peroxide.

The mutual effect of bacteria of the same or different species on one another is undoubtedly complex, and the frequent observation that a heavy inoculum into any slightly unsuitable medium, e.g., one containing dye, will grow when a smaller one containing a few healthy organisms will not, may depend on several

factors. But an important and possibly the only important element at work here is the presence of diffusible reducing substances which are primarily introduced with the inoculum later increasing with growth. As the first effect of many non-specific nucuous agents is to render bacteria hypersensitive to peroxide, growth or inhibition under their action is determined by the success or otherwise with which the peroxide, being continually produced under aerobic conditions, is kept at a low level. It is the function of the diffusible reducing substances to maintain this level, and their effectiveness will depend on:

1. The amount introduced with the inoculum.
2. The volume into which they diffuse during the earlier divisions of the organisms.
3. The degree of injury sustained by the bacteria.

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THE INFLUENCE OF CHOLESTEROL AND OXYCHOLESTEROL UPON THE MULTIPLICATION OF INFUSORIA *(ENCHELYS)*[1]

by

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It was discovered in 1913 by Robertson and Burnett (10) that cholesterol, emulsified in sodium oleate solution, greatly accelerates the growth of Flexner-Jobling carcinoma in rats when administered hypodermically in dosage of 40 mg. The rate of growth of the tumours in linear dimensions was from three to four times as rapid as that of the tumours in similar untreated animals, so that the growth in volume must have been from 27 to 84 times as rapid in treated animals as in the controls.

This fact was later confirmed by Sweet, Corson-White, and Saxon, who employed for this purpose a strain of Flexner-Jobling carcinoma which had never been known to yield metastases. The animals, after inoculation, were fed upon a diet to which cholesterol had been added to the extent of 200 milligrams per week. No less than 104 out of 116 animals which were thus treated developed metastases (14).

These observations immediately suggested two avenues of enquiry, the one into the effects of cholesterol upon other types of growth than that of carcinomatous tissue, the other into the effects of chemical relatives and modifications of cholesterol, with a view to ascertaining to what peculiarities of chemical configuration cholesterol owes its ability to accelerate the growth of carcinomata.

In pursuing the first of these two lines of enquiry Robertson and Ray (7, 12), have shown that cholesterol, so far from accelerating the growth of young animals (mice), actually induces very considerable retardation of growth, succeeded by a certain degree of compensatory acceleration, which, however, never enables the animals to reattain the dimensions of the controls. The effect of cholesterol upon the normal growth of mammals, therefore, is directly opposite to its effect upon the growth of carcinoma.

[1] The cost of publication of this article has been defrayed partly by the Animal Products Research Foundation.

The effect of cholesterol upon the multiplication of protozoa was investigated by Browder (1), who showed that comparing cultures of *Paramoecium*, each originally containing a single individual in which one-tenth of 1% of cholesterol had been introduced, with cultures in hay infusion without added cholesterol, after periods varying from one to three days the cholesterol cultures contained from 1·3 to 5·0 times as many individuals as the normal cultures. The effect of cholesterol upon the multiplication of these unicellular animals, therefore, is similar to its effect upon the growth of carcinoma.

The effect of various chemical relatives and derivatives of cholesterol upon the growth of Flexner-Jobling carcinoma was investigated by Robertson and Burnett (11), who showed that replacement of the hydroxyl group in cholesterol by acetyl, chlorine, or hydrogen, abolishes its capacity to accelerate the growth of the tumour tissue. Other hydroxy-benzene derivatives (meta-cresol, benzyl alcohol, hexahydrophenol, borneol, and inositol) were devoid of effect upon the growth of carcinoma.

It has been shown by Lifschutz that when cholesterol, dissolved in glacial acetic acid, is heated with benzoyl peroxide and the mixture poured into water, a substance is precipitated which yields the well-known deep violet colour with a mixture of strong sulphuric and glacial acetic acids, changing to blue upon the addition of ferric chloride (2). This substance, which is believed by Lifschutz to be a definite chemical unit, or a mixture of very closely related substances, and is termed by him oxycholesterol, has been shown to contain two hydroxyl groups instead of only one, as in cholesterol (5). Since the accelerative effect of cholesterol upon the growth of carcinoma has been shown by Robertson and Burnett to be dependent upon the hydroxyl group, the effect of a second hydroxyl group becomes of some importance to ascertain. Since the Flexner-Jobling carcinoma is unobtainable in Australia, I have investigated the effect of this substance upon the multiplication of infusoria *(Enchelys)*, since Browder's work, quoted above, showed that the effect of cholesterol upon the multiplication of another species of infusoria *(Paramoecium)* is similar to its effect upon the multiplication of the cells in carcinoma.

Oxycholesterol was prepared in accordance with the directions of Lifschutz and Grethe (6). Distinct evidence was obtained that oxycholesterol is not a single substance, but a mixture of substances of varying composition. Thus some preparations were soluble in water, yielding only slightly opalescent or soapy-looking solutions, while other preparations yielded emulsions in water which were nearly as opaque as emulsions containing equal concentrations of cholesterol. All of the preparations, however, yielded the Lifschutz reaction with sulphuric and acetic acids (2).

The experiments of Browder were repeated with *Enchelys*, and parallel experiments were performed in which oxycholesterol was added to the cultures instead of cholesterol. The method employed by Browder to emulsify the cholesterol in the culture-liquid was not found to be very satisfactory, only a fraction

of the cholesterol being actually emulsified. It was ascertained, however, that very stable emulsions of cholesterol may readily be prepared by delivering an alcoholic solution into ten times its volume hot water, with continual stirring. The alcohol may be removed from the emulsion by extraction with ether, which, curiously enough, does not remove the emulsified cholesterol, although it readily removes any portion of the cholesterol which may chance to have escaped emulsification. The ether may then be removed by heating the emulsion upon a water-bath and blowing air through it. Such emulsions have remained stable, depositing only a very small sediment, for over three years.

These emulsions,[2] and similar emulsions of oxycholesterol, were added in measured proportions to normal hay infusion, prepared by heating 5 grams of chaffed oat hay in 100 cc. of water on a boiling water-bath for one hour, and, after filtering, adding to the filtrate sufficient $\frac{N}{10}$ Na_2CO_3 to bring the mixture to a reaction suitable for the multiplication of infusoria (8). Single infusoria from the same parent culture were isolated into about 0·05 cc. of this infusion, alone and with the previous addition of cholesterol or oxycholesterol. The results of such experiments were all similar, and the following are illustrative:

Parent Cultures, A and B Series, three days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 24 hours.
99A	Hay Infusion	4
99B	Hay Infusion	4
100A	Hay Infusion + 0·03% Cholesterol	8
100B	Hay Infusion + 0·03% Cholesterol	8

Parent Culture, A' Series, three days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 24 hours.
101A	Hay Infusion	4
103A	Hay Infusion + 0·03% Cholesterol	16

Parent Culture, B Series, four days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 24 hours.
104B	Hay Infusion	4
106B	Hay Infusion + 0·03% Cholesterol	15

Parent Culture, A Series, three days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 24 hours.
108A	Hay Infusion	2
110A	Hay Infusion + 0·03% Cholesterol	8

[2] Usually, one volume of emulsion to nine of infusion.

Parent Cultures, A and B Series, three days old *(Euchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 24 hours.
125A	Hay Infusion	0
125B	Hay Infusion	8
127A	Hay Infusion + 0·04% Cholesterol	8
127B	Hay Infusion + 0·04% Cholesterol	16

Parent Cultures, A and B Series, six days old *(Euchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 24 hours.
129A	Hay Infusion	2
129B	Hay Infusion	4
130A	Hay Infusion + 0·08% Cholesterol	13
130B	Hay Infusion + 0·08% Cholesterol	16

Parent Cultures, A and B Series, two days old *(Euchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 24 hours.
134A	Hay Infusion	38
134B	Hay Infusion	27
135A	Hay Infusion + 0·08% Cholesterol	61
135B	Hay Infusion + 0·08% Cholesterol	45

Parent Cultures, A and B Series, four days old *(Euchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 24 hours.
163A	Hay Infusion	8
163B	Hay Infusion	8
164A	Hay Infusion + 0·04% Cholesterol	31
164B	Hay Infusion + 0·04% Cholesterol	32
165A	Hay Infusion + 0·04% Cholesterol	55
165B	Hay Infusion + 0·04% Cholesterol	41

The acceleration due to cholesterol is very striking, and fully confirms the previous findings of Browder. Nevertheless, the "lag-period" or period succeeding isolation of the parent individual, during which no multiplication occurs (8, 9), is not appreciably shortened by cholesterol. The acceleration is only observed after this period has elapsed, and for some time becomes progressively more evident. The following are illustrative experiments:

Parent Culture, A Series, three days old *(Euchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 18 hours.	21 hours.	24 hours.
101A	Hay Infusion	2	2	4
103A	Hay Infusion + 0·03% Cholesterol	2	3	16

Parent Culture, B Series, four days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 20 hours.	24 hours.
104B	Hay Infusion	2	4
106B	Hay Infusion + 0·03% Cholesterol ..	4	15

Parent Cultures, A and B Series, six days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 19 hours.	24 hours.
129A	Hay Infusion	1	2
129B	Hay Infusion	2	4
130A	Hay Infusion + 0·08% Cholesterol ..	3	13
130B	Hay Infustion + 0·08% Cholesterol ..	4	16

Hence in subcultures from old parent-cultures, in which the lag-period is very prolonged (8, 9), the accelerative effect of cholesterol may be small or absent after eighteen hours, and pronounced after twenty-two to twenty-four hours, as the following results show:

Parent Culture, A Series, four days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 18 hours.	21 hours.	24 hours.
122A	Hay Infusion	4	8	16
123A	Hay Infusion + 0·04% Cholesterol	4	8	25

When emulsions of oxycholesterol are added to the hay infusion, into which infusoria are cultured, the effect obtained varies somewhat with different preparations, further confirming the view, put forward above, that oxycholesterol is a variable mixture of substances. In every case, however, in which any acceleration of multiplication resulted, it was much less than the acceleration produced by an equal concentration of cholesterol. The following are illustrative results:

Parent Cultures, A and B Series, four days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 18 hours.	21 hours.	24 hours.
122A	Hay Infusion	4	8	16
122B	Hay Infusion	4	8	19
124A	Hay Infusion + 0·03% Oxycholesterol	4	8	32
124B	Hay Infusion + 0·03% Oxycholesterol	8	16	55

Parent Cultures, A and B Series, six days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 18 hours.	24 hours.
129A	Hay Infusion	1	2
129B	Hay Infusion	2	4
130A	Hay Infusion + 0·03% Cholesterol ..	3	13
130B	Hay Infusion + 0·03% Cholesterol ..	4	16
131A	Hay Infusion + 0·03% Oxycholesterol ..	2	7
131B	Hay Infusion + 0·03% Oxycholesterol ..	2	8

Parent Cultures, A and B Series, five days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 19 hours.	24 hours.
160A	Hay Infusion	2	4
160B	Hay Infusion	1	2
162A	Hay Infusion + 0·03% Oxycholesterol ..	2	6
162B	Hay Infusion + 0·03% Oxycholesterol ..	2	7

Parent Cultures, B Series, three days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 24 hours.
125B	Hay Infusion	8
126B	Hay Infusion + 0·04% Cholesterol	16
127B	Hay Infusion + 0·03% Oxycholesterol	1

Parent Cultures, A and B Series, two days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 18 hours.	20 hours.
138A	Hay Infusion	9	15
138B	Hay Infusion	8	14
139A	Hay Infusion + 0·03% Oxycholesterol ..	2	2
139B	Hay Infusion + 0·03% Oxycholesterol ..	4	7
140A	Hay Infusion + 0·06% Oxycholesterol ..	4	8
140B	Hay Infusion + 0·06% Oxycholesterol ..	4	8

It was at first suspected that the failure of oxycholesterol to induce acceleration of the multiplication of infusoria at all comparable with that produced by cholesterol might be attributable to impurities in the oxycholesterol, such as benzoic acid, derived from the reagents employed in its preparation. This uncertainty was, however, removed by the discovery that oxidation of cholesterol, with the production of a substance or substances yielding Lifschutz' reaction, may be brought about in the presence of certain substances by the mere passage of air through emulsions which are heated to boiling temperature.

Perfectly pure cholesterol does not undergo oxidation under these conditions, but emulsions which contain mere traces of substances derived from the acetone-extract of brain tissue are very readily oxidized in this fashion, and rapidly yield a violet colouration with sulphuric-acetic mixture. Upon evaporation such aerated emulsions yield a light-brown pasty mass resembling oxycholesterol, and the absorption spectrum of the fluid obtained after treating this residue with sulphuric-acetic mixture containing a trace of ferric chloride is similar to that yielded by oxycholesterol. We must infer that brain tissue contains a substance which is soluble in acetone and capable of catalyzing the oxidation of cholesterol by air at 100°C. The chemical characteristics of this catalyzer have not been further investigated, but its presence in brain tissue may possibly be related to the fact that preformed oxycholesterol is present in the brain (13).

Emulsions of cholesterol which contain this catalyzer exert the usual accelerative action upon the multiplication of *Enchelys*, but, after six or seven hours' aeration on a boiling water-bath, under a reflux condenser, the accelerative effect is either lost or considerably diminished:

Parent Cultures, A and B Series, five days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 19 hours.	24 hours.
160A	Hay Infusion	2	4
160B	Hay Infusion	1	2
161A	Hay Infusion + 0·02% aerated Cholesterol	2	4
161B	Hay Infusion + 0·02% aerated Cholesterol	2	4

Parent Cultures, A and B Series, four days old *(Enchelys)*.

Culture No.	Culture Fluid.	Number of Individuals in Culture after 19 hours.	24 hours.
163A	Hay Infusion	2	8
163B	Hay Infusion	2	8
165A	Hay Infusion + 0·04% Cholesterol ..	9	55
165B	Hay Infusion + 0·04% Cholesterol ..	7	41
166A	Hay Infusion + 0·04% same Cholesterol aerated	8	36
166B	Hay Infusion + 0·04% same Cholesterol aerated	4	22

The aerated emulsions employed in these experiments yielded Lifschutz' reaction, although, of course, it was not certain or even probable that the whole of the cholesterol was converted into oxycholesterol. An attempt was made to estimate the degree of conversion by colorimetric comparisons, but the lack of any definite standard of constant composition rendered the results of uncertain value. On the other hand, emulsions of cholesterol which failed to yield Lifschutz' reaction after aeration also displayed no loss whatever of power to accelerate the reproduction of infusoria.

It appears, therefore, that so slight a change in cholesterol as that induced by aeration in the presence of a suitable catalyzer at a temperature in the neighbourhood of 100°C. results in partial or total loss of its capacity to accelerate the reproduction of certain types of cells. This fact is the more remarkable because the observations of Lifschutz indicate that oxycholesterol is present in many mammalian tissues (3), and that defatted blood contains a catalyzer capable of converting cholesterol, dissolved in glacial acetic acid, into oxycholesterol (4). Presumably, therefore, the production of this substance constitutes the first step in the oxidation of cholesterol in the animal body, and is accompanied by the destruction of its physiological activity in relation to the growth of certain tissues.

These results, taken together with the previously cited results of Robertson and Burnett, indicate, although they do not yet suffice to prove, that the effect of cholesterol upon the reproduction of certain types of cells is peculiar to cholesterol itself, and that relatively slight changes in its chemical structure result in loss of its physiological activity.

CONCLUSIONS.

1. Stable emulsions of cholesterol may be prepared by discharging an alcoholic solution of cholesterol slowly into distilled water which is continuously stirred. The alcohol may be extracted with ether, which does not extract the emulsified cholesterol. The ether may be removed by heating and aeration.

2. Aeration of emulsions of pure cholesterol does not result in oxidation, but if traces of other acetone-soluble substances in brain tissue are present, aeration at boiling temperature rapidly oxidizes cholesterol emulsions with the production of a substance or substances resembling Lifschutz' oxycholesterol.

3. Oxidation of cholesterol in this manner, or by benzoyl peroxide in glacial acetic acid, diminishes, or even abolishes, its power to accelerate the multiplication of infusoria *(Enchelys)*.

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EXPERIMENTAL STUDIES ON GROWTH

XVII. THE PROPER METHOD OF GRAPHICALLY REPRESENTING CURVES OF GROWTH, AND THE APPLICABILITY OF THE LAW OF FORTUITOUS DISTRIBUTION TO THE INDIVIDUAL VARIATIONS IN THE WEIGHTS OF ANIMALS OF THE SAME GENERATION,[1]

by

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Observations on the technique of experiments on the growth of animals.

Previous communications of this series (7) have described the results of investigations upon the influence of substances, superadded to a varied and sufficient diet, upon the growth and longevity of white mice which were bred from an original stock of about fifty animals obtained from dealers in the United States of America. Our departure from Canada in 1919 necessitated the abandonment of this stock of animals, and early in 1920 four white mice (one male and three females) were obtained from Sydney through a local dealer. From these our present stock of animals has been derived, and they are all therefore descended from a single male. The intensive inbreeding thus involved has only been mitigated to this extent, that males and females from the same litters are not mated. It was hoped that by this means a very stable type might be produced, of low variability, both among themselves, in any given generation, and in the average dimensions of successive generations. This expectation has not, however, been fulfilled. Our animals are not less variable in weight than those which arose from our American stock, presumably of much more diverse origin.[2] Neither do successive generations resemble each other more closely than they did in our former stock (10), as we will show in subsequent communications of this series. The individual variations in size and weight of animals, therefore,

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[2] That is, the standard deviation varies from about 20 per cent. of the average weight in young and rapidly growing animals to about 10 per cent. of the average weight in adult animals, increasing again as the animals become senile.

appear not to be reducible by inbreeding, and we infer that they arise from spontaneous and inevitable variations in the germ plasm. In experiments designed to ascertain the effect of diet or environment upon the growth of animals, therefore, the normal dimensions at successive ages must be separately ascertained for the particular generation which is employed. We have not been able to discover, in other words, any fixed standard of weight for a given age which may be considered normal for the race, irrespective of the generation from which the individuals arise.

In our experiments we have adopted the conventionally approved plan of choosing normal and experimental animals alternately from the same litters. The tacit assumption which underlies this procedure is that the different members of the same litter differ from one another less, that is to say, are less variable than the population taken as a whole. The truth of this assumption has never, as a matter of fact, been established, and it would obviously be exceedingly difficult to do this, because the numbers constituting a litter of any species of mammal are too small to permit the accurate estimation of the variability of the members of the litter in terms of standard deviations. Nevertheless, our impression, gathered from the extremes of weight which may not infrequently be observed in members of the same litter, is that this assumption is unfounded, and that, in fact, members of the same litter are not less variable among themselves than the population as a whole. Nevertheless, we have continued to employ the procedure of taking normal and experimental animals alternately from the same litter because it offers the incidental advantage that the average number of individuals constituting the litters from which the animals are derived are by this means automatically equalized. Now we have observed that the number of animals which constitutes a litter very greatly modifies the early growth of its members. At the date of weaning the individuals constituting large litters are invariably smaller than those which constitute small litters. Their growth, up to this point and for some time later, is relatively retarded, and we have evidence, which we hope subsequently to present in detail, that such retardation in early life affects the whole life-history and subsequent growth of the animals. But a litter consisting of one animal, just because it develops more rapidly, is no more to be considered normal than a litter of eleven. There is no size of litter of which we can say that below this limit the animals are unaffected by the number of individuals comprising the litter, but whatever effect early nutritional deprivation or abundance or other conditions dependent upon the size of the litter may have upon the subsequent growth of the animals, they may be supposed to be substantially equal in litters of equal size. We assume that in all probability two groups of animals of any given generation would be alike, within the normal limits of variation, if they were chosen from litters of equal number although not the same. But choosing the animals alternately from the same litters affords an easy means of ensuring this equality, and in consequence we have adopted it

as a rule throughout our experiments, departing from the rule only in the small proportion of instances in which early deaths necessitate replacement by others of the animals which had been originally chosen for the experiment or for the controls.

The standard "varied and sufficient diet" which we have adopted in our experiments here differs for a variety of reasons from that which we used in our former experiments. In America we employed crushed oats as a staple, supplemented by hard biscuit and ½ cc. of mixed uncooked yolk and white of egg per mouse daily. Here we have employed, as the staple element of the diet, rice cooked in milk until nearly dry, supplemented by maize which is dipped into boiling water for one or two minutes and then allowed to dry, and 1 cc. of mixed uncooked white and yolk of egg per mouse daily. It is probable that the protein ration is somewhat higher than that which was received by the animals employed in our experiments in America.

The technique of the experiments was that which has been fully described in the first communication of this series (8). This technique is designed to eliminate epidemic infection altogether as a factor affecting the growth and welfare of the animals, and by the rigorous destruction of diseased stock and the employment of nests, cages, and breeding-boxes which permit frequent and efficient cleaning, and sterilization when necessary, we have so far succeeded in our object that deaths from epidemic infection, which had been reduced to less than 1% of all deaths in the experiments conducted in America, have in the experiments conducted here been reduced practically to zero.

During the first few weeks after removal of the animals from the breeding cages to the experimental cages, however, which involves the mixing of different litters, a small proportion of the animals develop an enteritis, accompanied, in some cases, by passage of blood, which is occasionally fatal. Those animals which lose weight and pass unformed faeces, or which pass blood, whether accompanied by loss of weight or not, are killed and replaced by others, and their weights are not included in the averages for the series. After about ten weeks of age this trouble is no longer encountered, and, as stated above, epidemic infection does not occur. We are not certain whether this enteritis in very young animals is actually due in the first place to infection, or whether it is not initiated by the change of diet involved in removal from the breeding-cages; for whereas the breeding stock receives chopped-up hard-boiled egg twice a week, the experimental animals, as we have stated, receive 1 cc. of mixed white and yolk of uncooked egg per animal daily, Sundays excepted.

The animals are weaned at three weeks of age and removed to the experimental cages at about five weeks of age. They are thereafter weighed weekly until they attain the age of seven months (210 days), and thereafter fortnightly. In estimating the average weight of the animals at different ages any weights shortly antedating death which are decidedly below or above the average preceding weight of the animal are excluded.

In each experiment 72 animals are employed of one sex, of which 36 are employed as controls and 36 receive the experimental additions to the diet, these additions being incorporated in the egg, which is administered to them six days per week. In order to ensure the complete consumption of the egg and its contents this is administered to the animals in the morning, before any other food is given to them, and except for the first three or four weeks, during which only part of the ration may be eaten, it is completely consumed in the twenty-four hours. Six animals inhabit one compartment of the cage, and receive 6 cc. of egg, which they share between them. While on the average it may be supposed that the animals receive 1 cc. apiece, in individual cases on certain days the dose may obviously vary from this average.

Objects of the experiments herein reported.

In the present paper we have to report the negative result of experiments initiated in 1921; but these results, although negative, are valuable, in that they demonstrate that different batches of animals chosen alternately from the same litters yield average weights which differ from one another by a less amount than the probable error throughout almost the whole duration of their lives. The positive results previously reported,[3] therefore, together with those which will subsequently be reported, are confirmed by the fact that appreciable deviations from the averages of the controls do not occur when the dietary additions are devoid of action upon the processes of growth.

In our previous experiments it had been found that cholesterol, when added to the diet of the white mouse in great excess (40 mg. per mouse per day) caused an initial retardation of growth of very considerable extent, followed by a compensatory acceleration, which, however, never enabled the animals to attain the dimensions of the controls. Since cholesterol is chemically a very inert substance, two alternative explanations of its action upon growth appear probable. Either the effect of cholesterol is due primarily to its peculiar physical properties, or

[3] These results may be briefly summarized as follows: The continuous administration of pituitary (anterior lobe) tissue to mice causes retardation of early (pre-adolescent) growth, followed by compensatory acceleration, which, however, never suffices to enable the animals to exceed the dimensions of the controls. The ether precipitable fraction from an alcoholic extract of pituitary anterior lobe tissue (Tethelin) in dosage of 4 milligrams per mouse per day causes similar but more pronounced deviation from normal growth and a decided increase of viability (duration of life 100 days in excess of normal). If, however, the administration of Tethelin is discontinued at about thirteen weeks of age, when retardation of growth is maximal, the subsequent compensatory acceleration is excessive, and leads to veritable gigantism of the animals (increase of 25 per cent. in average weight over that of normals) with, however, decreased rather than increased viability. Cholesterol, continuously administered in ten times the above dosage affects growth similarly to Tethelin, but without any increase of viability. Lecithin causes a continuous and uniform retardation of growth. Successive generations of animals yield curves of similar contour, but differing in amplitude to an extent greater than the probable error of the measurements. Long-lived animals differ characteristically from short-lived animals in growth. Brain-tissue, freed from cholesterol and other substances soluble in acetone, is devoid of influence upon the growth of mice.

the effect of cholesterol is due to a small proportion of the cholesterol so altered in the body as to become of a much more reactive character. Since any changes which cholesterol may undergo in the body will most probably be due to oxidation, we felt it advisable to investigate various oxidation-products of cholesterol with a view to ascertaining whether any of these might chance to have a much more powerful influence upon growth than cholesterol itself.

It has been shown by Westphalen (12) that when cholesterol is treated with benzoyl peroxide in boiling chloroform one atom of oxygen is taken up with the formation of a crystalline compound (α-cholesteryl oxide), which, however, like cholesterol itself, contains only one hydroxyl group, in this respect differing from Lifschutz' "oxycholesterol," which is a mixture of substances containing more than one hydroxyl group (5, 6). A considerable quantity of α-cholesteryl oxide was prepared and administered to a group of 36 male mice. The arbitrary dosage of 5 mg. per mouse per day was employed, because it was considered that if oxidation resulted in the production of any substance very much more potent in affecting growth than cholesterol itself, such a substance might be expected to be not less than eight times as potent as cholesterol, more particularly having regard to the fact that a very large proportion of the cholesterol which is actually absorbed when administered to animals is stored away apparently unaltered in various tissues (1, 2, 3).

The oxycholesterol of Lifschutz, which contains two hydroxyl groups, was not employed because, as shown in the preceding paper, it had already been ascertained, before these experiments were initiated, that this method of oxidation deprives cholesterol of its power to accelerate the reproduction of infusoria, and therefore, presumably, its power of influencing other types of growth as well.

When impure α-cholesteryl oxide, which is still contaminated with benzoyl peroxide, is heated upon a boiling water-bath an explosive reaction occurs, with the evolution of white fumes of benzoic acid. The brown pasty residue consists of a mixture of substances which are soluble in alkaline water, and yield a reversed Salkowski reaction (red in the sulphuric acid layer) similar to that yielded by the bile acids. This substance was administered to female mice in the same arbitrary dosage of 5 mg. per day per mouse.

The results, as the accompanying tables show (Tables 1-4), were completely negative, since within the probable errors of the average weights, the experimental and control animals were identical; the results are also graphically displayed in figures 2 and 3, which are constructed in a manner not hitherto employed in investigations of this type, and which depends upon the following principles:

Explanation of the graphic method employed.

In estimating the mean value of any measurable dimension of fortuitously variable material, the value which we obtain by averaging the variable results

is not properly representable as a point, but as an area, within which the true value is as likely as not to lie. The distance from the centre of this area to its periphery is the "probable error" of the estimation. This may be estimated in the usual manner employed by statisticians, by dividing the "standard deviation" by the square root of the number of measurements, and multiplying this result by the factor 0·6745, that is,

$$\text{p.e.} = 0{\cdot}6745\frac{\sigma}{\sqrt{N}}$$

The "standard deviation" of any series of measurements is the deviation from the average within which two-thirds of all of the observed deviations will fall.[4] It is ascertained by adding the squares of all observed deviations from the average and dividing this sum by the number of measurements so as to obtain the mean square of the deviations. The square root of the mean square of the observed deviations is the "standard deviation" of the series.

If we are estimating the alteration of variable dimensions, such as weights or lengths of animals with expiry of time, the fortuitous error in the estimation of time is practically negligible, and the area which is actually determined reduces to a straight line, of which the length is twice the "probable error" of the average, the centre being situated at that point in the diagram which corresponds to the ascertained average. If at stated intervals of time a series of such measurements is made, a corresponding series of lines will be obtained, varying in width as the probable error varies and in situation as the growth of the animal proceeds. If the extremities of these lines are joined by two continuous curves, these curves will enclose a narrow area, portraying in its width the variations of the "probable errors" of the measurements. The true curve of growth will obviously lie, as likely as not, wholly within this area.

Applying this method of graphic portrayal to the measurements enumerated in Table 1, we obtain the diagram shown in figure 1, which displays all we are actually entitled to infer from the measurements. To portray such results as they are usually portrayed, in the form of a series of points (i.e., averages), united by a continuous line, is misleading, for it conveys no idea of the degree of deviation from this curve which another batch of precisely similar animals might be just as likely to display as not. All probable curves, that is, one-half of all possible curves obtainable from other batches of similar material, are, however, simultaneously portrayed in such a diagram as that in figure 1, and we can at a glance perceive what deviations from the average weight at any age are simply fortuitous, i.e., as likely as not to occur, and what deviations may certainly be regarded as attributable to systematic causes, i.e., to differences in the genetic constitution of the animals themselves, or to differences of diet or environment. The actual lines corresponding to twice the probable errors of each average are not shown in the diagram, but their positions are indicated by the extremities of the shading and their length by the vertical width of the area at any given age.

[4] More accurately 68·27%.

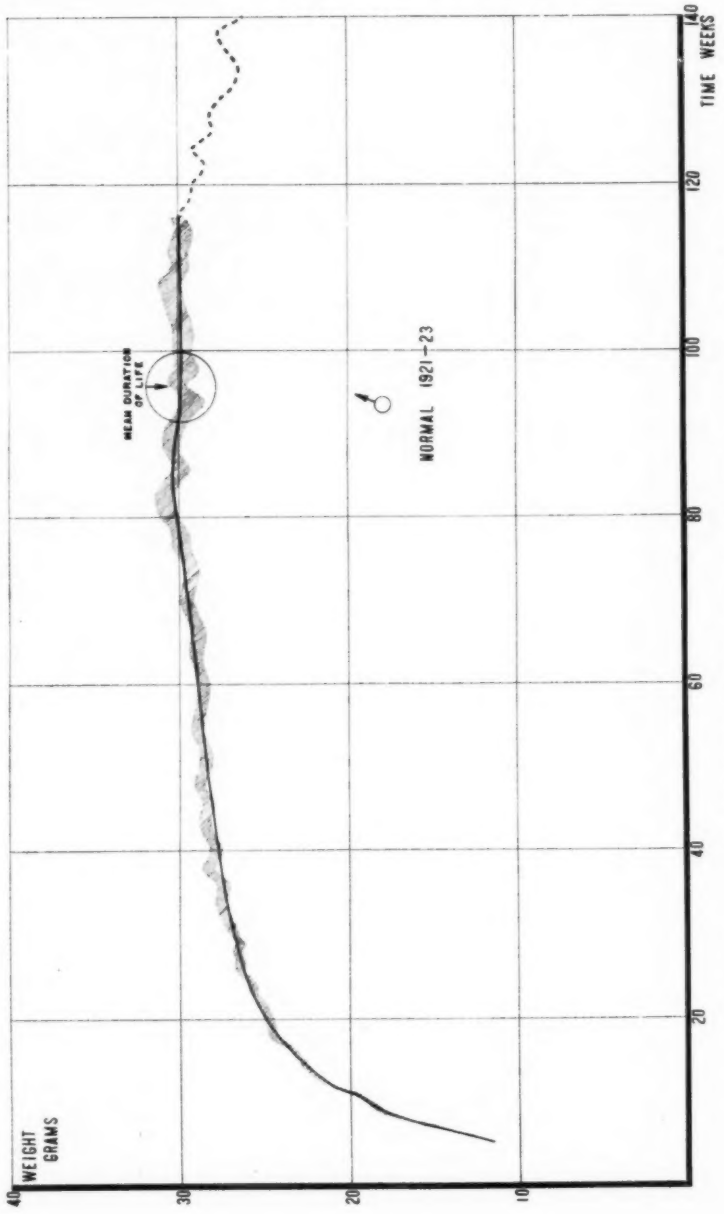

Fig. 1. The Growth of Normal Male White Mice, 1921-23. The vertical width of the shaded area is twice the probable error of the average weight. The smooth curve is a curve of autocatalysis rotated through an angle of 8°. The mean duration of life is indicated by the centre of the circle, of which the radius is the probable error of the estimation.

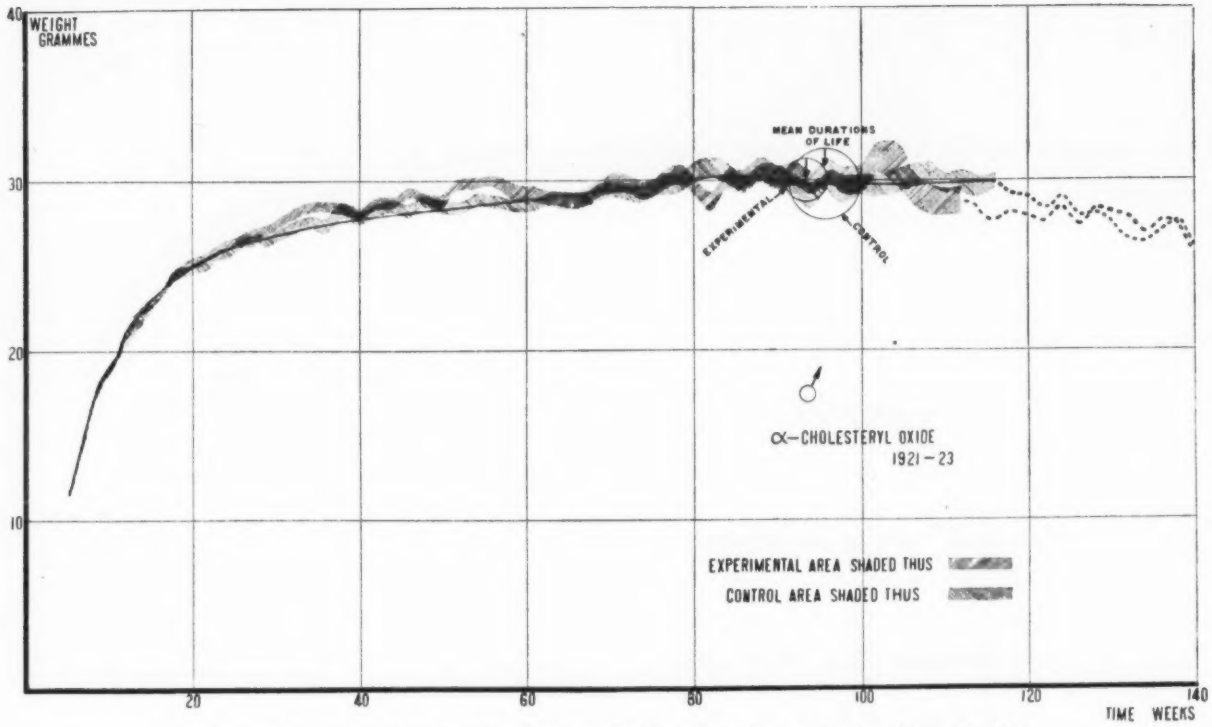

Fig 2. Comparison of the Growth of Male White Mice receiving 5 mg. of α-cholesteryl oxide per day with that of Normal Male White Mice during the same period (1921-23). The two shaded areas overlap for almost the whole duration of life. The growth of the two groups of animals may therefore be considered to have been identical. The circles indicating the probable errors of the mean durations of life also intersect. The durations of life of the two groups are therefore also to be considered identical.

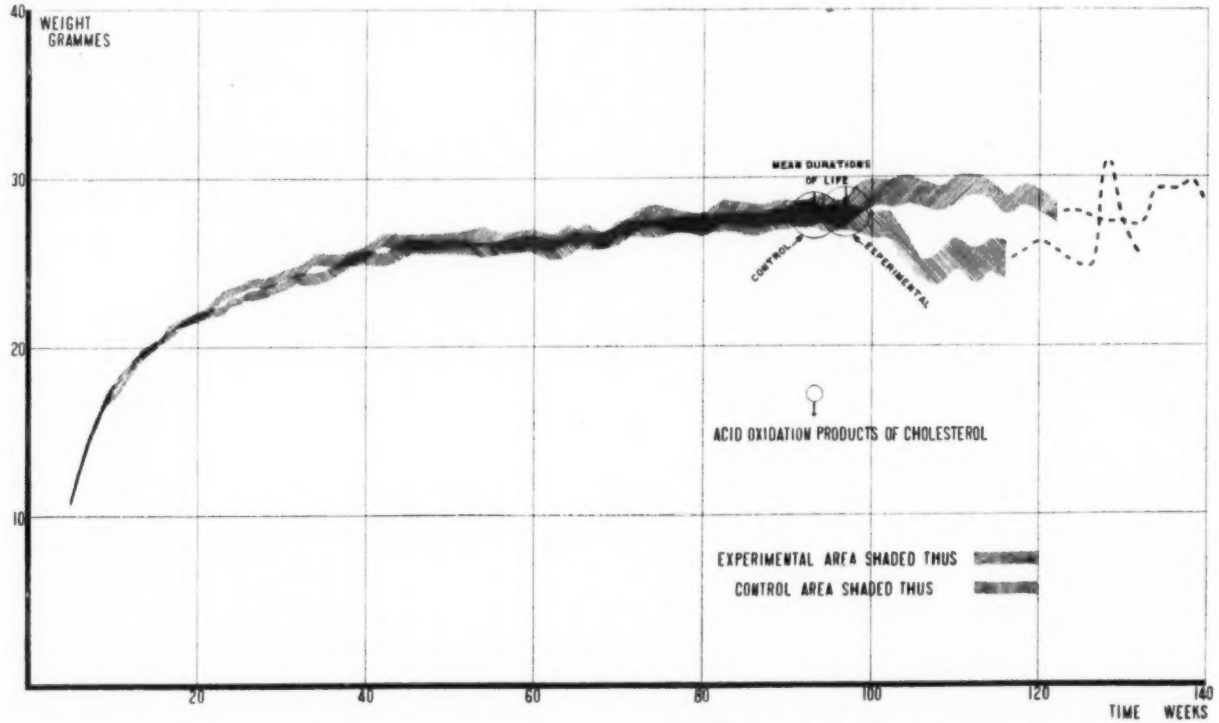

Fig. 3. Comparison of the Growth of Female White Mice receiving 5 mg. of acid oxidation products of Cholesterol per day, with that of Normal Female White Mice during the same period (1921-23). The two shaded areas overlap until the mean duration of life has been exceeded and the animals, now senile, have become characteristically unstable and susceptible to slight environmental fluctuations. The growth of the two groups of animals may therefore be considered to have been identical. The circles indicating the probable errors of the mean durations of life also intersect. The durations of life of the two groups are therefore also to be considered identical.

The type of growth-chart, which is ordinarily employed, is also misleading for another reason. With alterations of age the variability of the animals also alters, being high in young animals, decreasing as the rate of growth increases, and increasing again in old age, when the weight of the animals becomes very unstable and very susceptible to slight fluctuations of the environment (10, 11). Moreover, in experiments extending, as these do, over the whole duration of the lives of the animals, the number of animals subjected to measurement necessarily diminishes after a certain age, and decreases ultimately to zero. The averages determined at different ages are therefore of very different value, possessing a high degree of certainty at one age and little at another. In diagrams such as that portrayed in figure 1, the degree of certainty attaching to each measurement is ascertainable at a glance. It will be observed that the correct position of the curve of growth becomes more uncertain as the age of the animals increases, mainly on account of the progressive diminution in the number of the survivors.[5] The high comparative variability of young animals does not prevent the early part of the curve from being the most certain, because at these ages growth is so rapid that a relatively considerable departure from the average weight corresponds only to a very slight displacement of the curve along the time-axis.

At the termination of the shaded area a broken line shows, in the manner usually employed, the average weights of the surviving animals.[6] The shaded area ceases at the moment when the number of survivors has fallen to five, because after this the probable error of the estimation of the probable error itself becomes so large as to render its computation of little or no value. At this point, in fact, uncertainty becomes so great that we can no longer form any estimate of its magnitude.

The contour of the area reveals certain fluctuations. Of these the first considerable fluctuation, occurring at about ten weeks of age, is found in about the same situation in every group of mice which has been investigated, here and in America. It is a constant characteristic of the growth of these animals, and evidently represents the junction of two cycles of growth (9). The subsequent fluctuations are irregular in situation, and a combination of two curves from separate groups of similar animals (as in figures 2 and 3) results in the cancellation of the majority of them through the frequent coincidence of the maxima of one curve with the minima of the other. We may infer that these later fluctuations are accidental, and owe their origin to unperceived fluctuations of

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[5] Strictly speaking, the number of animals measured rather than the number of animals surviving. Animals, especially the older ones, generally lose or gain weight rather suddenly for two or three weeks prior to death, and in estimating average weights such sudden premortal gains or losses are excluded. The "number of animals" indicated in Tables 1 to 4, therefore, may differ from the true "number of survivors" by one or two animals or by two or three weeks.

[6] In the broken curve the sudden premortal changes of weight alluded to above have not been omitted, since in very old animals they are highly characteristic and more prolonged than in younger animals.

the environmental conditions. They become very much more pronounced in the older animals, especially after the expiry of the average duration of life, and reveal the instability which is characteristic of senile animals (10, 11).

Through the shaded area in figure 1 is drawn a continuous "smoothed curve" to show what this would look like were the ordinary method of representation of such results employed. The curve is of the general type previously found to represent the growth of normal mice. This curve subsequently to the tenth week and until the attainment of the maximum, which just anticipates the normal duration of life, was drawn by projecting upon the shaded area a series of curves of autocatalysis with different velocity constants. The curve which fitted the shaded area most closely is that represented. It is clear that a curve of autocatalysis will lie almost wholly within the shaded area, in other words, that the relationship of growth to time is capable of representation by the autocatalytic formula. It was found necessary, however, in order to fit any curve at all, to rotate the projected curves through an angle of 8°, indicating the occurrence of a very slow and approximately linear accretion of weight ($dw = 0{\cdot}07dt$), which is not expressed by the autocatalytic formula. This has been found to be characteristic of all curves of growth yielded by mice, and represents either an extremely slow autocatalytic process, which would very closely imitate a linear process, or a truly linear accretion of weight, which proceeds concurrently with the main growth-process, which is of the ordinary autocatalyzed type.

Since the vertical width of the shaded area in these diagrams represents the limits of deviation within which the true curve of growth may as likely as not be found to lie, any similarly constructed area, yielded by any equally numerous group of precisely similar animals, should overlap the first area through the greater part of its extent (accidental fluctuations excluded). On the other hand, if the growth of the second group of animals differed from that of the first group, the two areas should, continuously and systematically, diverge from one another, the extent of such divergence, of course, being an indication of the extent of the systematic difference between the two groups, to whatever cause such difference might owe its origin. Of course, this would be necessarily the case only if the deviations from the average weight which are displayed by individual animals are of entirely fortuitous origin and distributed in the manner indicated by Gauss's "curve of probability," as assumed in the application of the formulae for standard deviation, probable error, etc., derived from the properties of that curve.

In figure 2 are simultaneously displayed the areas corresponding to the growth of normal males during the period 1921-23, and the growth of those males which received α-cholesteryl oxide in their diet. The two areas overlap almost throughout their whole extent, the very brief separations of the areas evidently being attributable to accidental fluctuations of contour. We may infer from this, *firstly*, that α-cholesteryl oxide, in the dosage employed, is devoid of

measurable effect upon the growth of white mice, and, *secondly*, that the laws of fortuitous distribution apply to the deviations from average weight which are encountered in individual mice. In figure 3 are similarly displayed the corresponding areas for normal females during the same period, and those females which received the acid oxidation products of cholesterol. The areas similarly overlap through almost the whole of their extent, the only significant separation of the curves occurring after the mean duration of life, when less than half of the animals were surviving, and these senescent and characteristically susceptible to environmental influences. It is questionable whether any real significance can be attached to separation of the areas at this late date, more especially since the probable errors of the durations of life overlap, that is, the mean duration of life in the two groups does not differ to any appreciable degree. We may therefore infer, as in the case of the males, that the substance administered, in the dosage employed, was without effect upon the growth of the animals, and, again, that the law of fortuitous distribution of variates applies to the deviations of the weights of these animals from the average.

The technique employed in these experiments is therefore adequate, within the ascertained magnitude of the probable error, to permit reduplication of results by two simultaneously selected batches of normal animals. It is therefore adequate to establish with certainty systematic deviations of growth from the normal, due to peculiarities of diet or environment, such as those which have been reported in previous communications of this series.

Mortality statistics.

The mortality statistics for the mice employed in these experiments are enumerated in Tables 5 to 8 inclusive. These may be summarized as follows:

The average duration of life both of males and females is between 650 and 670 days, and of those animals which survive at 210 days of age one-fourth will have died at the expiration of 500 days, one-third at 600 days, one-half at 700 days, and three-fourths at 800 days. There is little difference, if any, between the viability of males and females in this series, although occasionally males in the proportion of about 3 in 100 survive to over 1,000 days of age, which was attained by no female among those employed in these experiments.

In computing these mortality statistics "accidental deaths" are excluded. These occur almost wholly among male animals, and are attributable to their belligerent habits. As a result of fighting extensive lacerations are not uncommon, and sometimes these, or internal injuries received in the same fashion, lead to death of the animal. All deaths prior to 210 days, these forming only a very small proportion, are regarded as "accidental," since, in our experience, such early deaths are either attributable to fighting among males or to very occasional mechanical injuries received in the cages, or to the early enteritis alluded to above. On the other hand, deaths from local infections, which occasionally

develop late in life in consequence of early injuries (in males), are included in the tables, since such infections, which have remained latent for the greater part of the duration of life, would not lead to the death of the animals were it not for the general senescent deterioration of tissues, which ultimately permits the infection to gain headway.

These results differ to a certain extent from those reported in our previous experiments in America. The viability of our present stock is appreciably less than that of our American stock, of which the average duration of life was 767 days for males, with a probable error of 24 days, and 719 days for females, with the same probable error. This inferiority of viability is revealed, not only in the average duration of life, but also in the diminished expectation of life during each age period. Since our diet at present employed would appear to be no less "varied and sufficient" than that formerly supplied to our animals in America, this lessened viability may be an expression of the differing genetic constitution of the two stocks of animals; or it may represent a consequence of the higher plane of protein intake in our present experiments, attributable to the employment of rice cooked in milk instead of crushed oats, as the staple element of the diet (4).

The mean durations of life are indicated in figures 1, 2, and 3 by circles, of which the centres are situated at that point in the middle of the growth area which corresponds to the ascertained average life-durations, while the radii of the circles are equal to the probable errors of these averages. Intersection of two circles, as in figures 2 and 3, obviously implies that the two life-durations are identical within the probable errors of the estimates. Had the growth areas diverged appreciably at this point it would have been preferable to indicate the durations of life upon a separate line drawn parallel to the time-axis, for otherwise two circles representing genuinely identical life-durations might fail to intersect simply because of their differing distances from the base-line.

As regards the causes of death, since epidemic infections are excluded by our technique, they are confined, apart from occasional local infections of the urethra, etc., in old males, to those disease conditions which are characteristic of old age in all animals, namely, neoplasms, degenerative changes in the heart, kidneys, and liver, and senile atrophy. Our present stock differs greatly from our American stock in the almost total absence of carcinoma, which only rarely appears here, while in our American experiments it accounted for from 25% to 50% of the total deaths.[7] On the other hand, Lymphadenomas and Sarcomas are very abundant, and claim an even larger proportion of victims than they did in our experiments formerly reported.

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[7] It has been shown in a previous communication (Journal of Cancer Research, 6 (1921), p. 7) that animals which ultimately develop carcinoma grow in a manner characteristic of long-lived animals. The lower viability of our present stock of animals, therefore, in comparison with our American stock, may be correlated with the comparative absence of carcinoma.

SUMMARY OF RESULTS.

1. The technique of experiments upon mice, described in previous communications of this series, is further elaborated in this paper. This technique achieves the total exclusion of epidemic death as a factor affecting the growth or viability of the animals.

2. A new method of graphic portrayal of the growth of animals is described. The increase of weight with time is represented by an area, of which the middle point at any given age is the ascertained average weight, while the width of the area is twice the "probable error" of the average. When two such areas overlap during the greater part of their extent, and such divergences as occur are brief and irregular, the growth-curves of the two groups of animals may be considered to be identical. The duration of life is similarly represented by a circle, of which the centre is situated at the ascertained average age of death, and the radius is equal to the "probable error" of this average. When two such circles overlap, the average duration of life of the two groups of animals may be considered to be identical.

3. Animals which received 5 mg. per day of α-cholesteryl oxide, and animals which received the same dosage of the acid products resulting from the further oxidation of α-cholesteryl oxide with benzoyl peroxide, did not differ, either in growth or in longevity, from normal animals of the same generation.

4. The technique employed in these experiments is therefore adequate, within the ascertained magnitudes of the probable errors, to permit reduplication of results by two simultaneously selected batches of normal animals. It is therefore adequate to establish with certainty systematic deviations of growth from the normal, due to peculiarities of diet or environment, such as those which have been reported in previous communications of this series (Journal of Biological Chemistry, Vols. XXIV, XXV, XXVII, XXXI, XXXVII, XLII, and XLIV).

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TABLE 1.

GROWTH OF NORMAL MALE WHITE MICE, 1921-23.

No. of Animals.	Age in weeks.	Mean weight in grammes.	Probable error of the mean.
33	5	11·27	± 0·29
35	6	12·97	± 0·32
35	7	14·91	± 0·32
35	8	16·77	± 0·30
35	9	18·17	± 0·28
35	10	18·80	± 0·28
35	11	19·80	± 0·30
35	12	21·16	± 0·30
35	13	21·80	± 0·29
35	14	22·46	± 0·29
35	15	22·84	± 0·30
35	16	23·19	± 0·30
35	17	23·83	± 0·31
35	18	24·51	± 0·31
35	19	24·66	± 0·30
35	20	24·76	± 0·30
35	21	24·97	± 0·28
35	22	25·17	± 0·26
34	23	25·65	± 0·27
34	24	25·69	± 0·32
34	25	26·06	± 0·34
33	26	26·48	± 0·35
33	27	26·48	± 0·32
33	28	26·68	± 0·29

No. of Animals.	Age in weeks.	Mean weight in grammes.	Probable error of the mean.
33	29	26·33	± 0·31
33	30	26·77	± 0·31
32	32	27·16	± 0·36
30	34	27·63	± 0·34
27	36	27·31	± 0·43
28	38	28·32	± 0·42
27	40	27·74	± 0·41
27	42	28·26	± 0·38
27	44	28·50	± 0·38
27	46	28·17	± 0·39
26	48	28·81	± 0·36
22	50	28·45	± 0·39
22	52	28·36	± 0·39
21	54	28·88	± 0·40
21	56	28·79	± 0·39
21	58	28·60	± 0·37
20	60	28·45	± 0·34
20	62	28·75	± 0·31
18	64	28·97	± 0·45
18	66	28·75	± 0·45
17	68	29·21	± 0·48
17	70	29·47	± 0·44
15	72	29·27	± 0·48
14	74	29·18	± 0·47
13	76	29·73	± 0·59
13	78	29·77	± 0·59
12	80	30·50	± 0·56
12	82	30·75	± 0·64
12	84	30·42	± 0·62
12	86	29·67	± 0·49
11	88	30·36	± 0·57
11	90	30·23	± 0·59
10	92	29·65	± 0·64
10	94	28·95	± 0·79
10	96	30·00	± 0·55
10	98	29·40	± 0·81
10	100	29·65	± 0·57
10	102	29·60	± 0·64
8	104	29·88	± 0·70
7	106	30·21	± 0·61
7	108	30·43	± 0·69

No. of Animals.	Age in weeks.	Mean weight in grammes.	Probable error of the mean.
7	110	29·79	± 0·54
6	112	29·92	± 0·68
6	114	29·58	± 0·61
6	116	29·92	± 0·47
5	118	29·20	—
4	120	29·00	—
4	122	28·25	—
4	124	29·00	—
3	126	27·83	—
3	128	28·00	—
3	130	27·50	—
2	132	26·50	—
2	134	26·25	—
1	136	27·00	—
1	138	27·50	—
1	140	26·00	—
1	142	27·50	—
1	144	26·50	—
1	146	26·00	—
1	150	27·00	—
1	152	27·00	—
1	154	26·50	—

TABLE 2.

GROWTH OF MALE WHITE MICE RECEIVING α-CHOLESTEROL OXIDE, 1921-23.

No. of Animals.	Age in weeks.	Mean weight in grammes.	Probable error of the mean.
35	5	11·37	± 0·26
35	6	13·71	± 0·28
36	7	14·96	± 0·27
36	8	16·89	± 0·27
36	9	18·08	± 0·29
36	10	19·11	± 0·25
36	11	19·82	± 0·23
36	12	20·60	± 0·25
35	13	21·27	± 0·24
35	14	21·91	± 0·27
35	15	22·73	± 0·29
35	16	23·33	± 0·31
35	17	23·83	± 0·28

No. of Animals.	Age in weeks.	Mean weight in grammes.	Probable error of the mean.
35	18	24·50	± 0·27
35	19	25·01	± 0·28
35	20	25·21	± 0·30
35	21	25·41	± 0·29
35	22	25·67	± 0·29
35	23	25·96	± 0·28
35	24	26·29	± 0·29
35	25	26·39	± 0·29
35	26	26·74	± 0·31
35	27	26·84	± 0·27
35	28	27·11	± 0·28
35	29	27·17	± 0·31
35	30	27·34	± 0·33
35	32	28·04	± 0·29
35	34	28·46	± 0·33
35	36	28·53	± 0·34
35	38	28·41	± 0·32
35	40	28·11	± 0·29
35	42	28·79	± 0·33
35	44	28·71	± 0·35
35	46	29·36	± 0·32
35	48	28·97	± 0·35
35	50	28·76	± 0·36
35	52	29·84	± 0·32
35	54	29·83	± 0·33
34	56	30·00	± 0·37
34	58	29·43	± 0·41
34	60	29·31	± 0·43
32	62	29·09	± 0·42
30	64	28·82	± 0·51
30	66	28·88	± 0·51
28	68	29·30	± 0·49
27	70	29·98	± 0·47
27	72	29·85	± 0·50
26	74	29·35	± 0·52
26	76	29·83	± 0·53
26	78	30·38	± 0·54
25	80	29·62	± 0·56
23	82	28·65	± 0·58
22	84	30·02	± 0·60
20	86	29·95	± 0·61

No. of Animals.	Age in weeks.	Mean weight in grammes.	Probable error of the mean.
19	88	30·66	± 0·69
18	90	30·11	± 0·70
17	92	30·44	± 0·70
17	94	29·79	± 0·63
17	96	30·50	± 0·71
15	98	29·87	± 0·77
13	100	30·08	± 0·87
12	102	31·29	± 0·90
8	104	30·94	± 1·30
6	106	29·58	± 0·94
6	108	28·75	± 0·86
6	110	28·58	± 0·80
6	112	28·75	± 0·78
5	114	28·90	—
4	116	27·50	—
4	118	28·00	—
4	120	27·75	—
4	122	27·38	—
3	124	28·33	—
2	126	27·25	—
2	128	28·25	—
2	130	28·00	—
2	132	27·75	—
2	134	26·75	—
2	136	27·50	—
2	138	27·25	—
2	140	25·75	—
2	142	24·75	—
1	144	24·50	—
1	146	22·00	—

TABLE 3.

GROWTH OF NORMAL FEMALE WHITE MICE, 1921-23.

No. of Animals.	Age in weeks.	Mean weight in grammes.	Probable error of the mean.
27	5	10·57	± 0·27
28	6	12·23	± 0·27
28	7	14·05	± 0·27
28	8	15·43	± 0·25
28	9	16·52	± 0·27

No. of Animals.	Age in weeks.	Mean weight in grammes.	Probable error of the mean.
28	10	16·98	± 0·23
28	11	17·59	± 0·25
28	12	18·34	± 0·26
28	13	19·07	± 0·27
28	14	19·59	± 0·23
28	15	20·02	± 0·23
28	16	20·45	± 0·23
28	17	20·73	± 0·23
28	18	21·21	± 0·22
28	19	21·46	± 0·23
28	20	21·70	± 0·23
28	21	21·80	± 0·25
28	22	22·09	± 0·23
28	23	22·14	± 0·26
28	24	22·73	± 0·27
28	25	22·70	± 0·28
28	26	23·05	± 0·29
28	27	23·05	± 0·32
28	28	23·23	± 0·33
28	29	23·41	± 0·32
28	30	23·54	± 0·30
28	32	24·04	± 0·34
28	34	24·05	± 0·39
28	36	24·43	± 0·36
28	38	25·09	± 0·40
28	40	25·36	± 0·43
28	42	25·23	± 0·42
27	44	25·69	± 0·45
27	46	26·00	± 0·45
27	48	25·93	± 0·40
27	50	26·00	± 0·45
27	52	25·94	± 0·41
26	54	25·79	± 0·42
26	56	25·90	± 0·40
25	58	26·06	± 0·47
25	60	26·14	± 0·53
22	62	25·80	± 0·67
22	64	25·98	± 0·62
22	66	26·77	± 0·59
22	68	26·34	± 0·52
21	70	27·05	± 0·47

No. of Animals.	Age in weeks.	Mean weight in grammes.	Probable error of the mean.
21	72	27·05	± 0·49
21	74	27·00	± 0·57
21	76	27·17	± 0·48
21	78	27·26	± 0·49
21	80	27·29	± 0·52
19	82	27·32	± 0·53
19	84	27·39	± 0·62
19	86	27·21	± 0·68
18	88	27·50	± 0·67
15	90	27·60	± 0·68
15	92	27·93	± 0·77
13	94	27·65	± 0·76
12	96	27·63	± 0·79
12	98	27·54	± 0·80
10	100	27·25	± 0·79
10	102	27·25	± 0·83
9	104	25·83	± 0·91
8	106	24·56	± 0·59
7	108	24·64	± 1·01
6	110	25·58	± 0·94
6	112	24·83	± 1·07
6	114	25·42	± 0·98
5	116	25·10	—
3	118	25·67	—
2	120	26·25	—
2	122	25·75	—
2	124	25·00	—
2	126	24·75	—
1	128	31·00	—
1	130	27·50	—
1	132	25·50	—

TABLE 4.

GROWTH OF FEMALE WHITE MICE RECEIVING ACID OXIDATION PRODUCTS OF CHOLESTEROL, 1921-23.

No. of Animals.	Age in weeks.	Mean weight in grammes.	Probable error of the mean.
35	5	10·90	± 0·23
36	6	12·63	± 0·23

No. of Animals.	Age in weeks.	Mean weight in grammes.	Probable error of the mean.
36	7	14·10	± 0·24
36	8	15·29	± 0·23
36	9	16·53	± 0·25
36	10	17·50	± 0·22
35	11	18·26	± 0·22
33	12	18·80	± 0·24
31	13	19·44	± 0·24
31	14	19·76	± 0·26
31	15	20·11	± 0·25
31	16	20·66	± 0·26
31	17	21·23	± 0·25
31	18	21·50	± 0·25
31	19	21·65	± 0·23
31	20	21·89	± 0·24
31	21	22·05	± 0·24
31	22	22·63	± 0·25
31	23	23·03	± 0·26
31	24	23·29	± 0·27
31	25	23·44	± 0·26
31	26	23·74	± 0·29
31	27	23·84	± 0·27
31	28	23·79	± 0·28
31	29	24·05	± 0·30
31	30	24·27	± 0·32
31	32	24·50	± 0·33
31	34	25·24	± 0·34
31	36	25·19	± 0·38
31	38	25·32	± 0·38
31	40	25·68	± 0·40
30	42	26·43	± 0·41
30	44	26·33	± 0·43
30	46	26·32	± 0·46
30	48	26·33	± 0·45
30	50	26·37	± 0·47
30	52	26·48	± 0·49
30	54	26·65	± 0·50
29	56	26·33	± 0·52
29	58	26·40	± 0·57
28	60	26·45	± 0·54
28	62	26·20	± 0·55

No. of Animals.	Age in weeks.	Mean weight in grammes.	Probable error of the mean.
27	64	26·76	± 0·48
27	66	26·28	± 0·46
26	68	26·31	± 0·50
26	70	26·71	± 0·52
26	72	27·52	± 0·54
26	74	27·83	± 0·60
26	76	27·63	± 0·62
25	78	27·44	± 0·61
22	80	27·27	± 0·67
22	82	28·05	± 0·71
22	84	28·00	± 0·67
21	86	27·81	± 0·69
21	88	27·93	± 0·68
20	90	28·05	± 0·69
18	92	27·89	± 0·84
17	94	28·29	± 0·80
17	96	27·88	± 0·82
15	98	28·60	± 0·92
14	100	29·14	± 0·78
13	102	29·08	± 0·77
12	104	29·33	± 0·82
12	106	29·13	± 0·76
11	108	28·82	± 0·70
8	110	29·38	± 0·81
7	112	29·64	± 0·61
7	114	29·36	± 0·49
7	116	28·43	± 0·58
5	118	29·10	—
5	120	28·70	—
5	122	28·00	—
4	124	28·13	—
3	126	27·83	—
3	128	27·50	—
3	130	27·50	—
3	132	27·33	—
2	134	29·50	—
1	136	29·50	—
1	138	30·00	—
1	140	28·50	—
1	142	28·50	—

TABLE 5.

MORTALITY STATISTICS FOR NORMAL MALE WHITE MICE, 1921-23.

(Accidental deaths excluded.)

Age Period.	Per cent. of all animals dying within this period.	Per cent. of survivors dying within this period.	Per cent. of all animals dead at end of this period.
210–500 days	28·5	28·5	28·5
500–600 ,,	14·3	20·0	42·8
600–700 ,,	9·5	16·6	52·3
700–800 ,,	19·1	40·0	71·4
800–900 ,,	14·3	50·0	85·7
900–1000 ,,	9·5	66·7	95·2
1000–1100 ,,	4·8	100·0	100·0

Mean duration of life, 670 ± 29 days.

TABLE 6.

MORTALITY STATISTICS FOR MALE WHITE MICE RECEIVING *a*-CHOLESTEROL OXIDE, 1921-23.

(Accidental deaths excluded.)

Age Period.	Per cent. of all animals dying within this period.	Per cent. of survivors dying within this period.	Per cent. of all animals dead at end of this period.
210–500 days	22·8	22·8	22·8
500–600 ,,	17·1	22·2	39·9
600–700 ,,	17·1	28·6	57·0
700–800 ,,	25·6	60·0	82·6
800–900 ,,	11·4	66·7	94·0
900–1000 ,,	3·0	50·0	97·0
1000–1100 ,,	3·0	100·0	100·0

Mean duration of life, 654 ± 18 days.

TABLE 7.

MORTALITY STATISTICS FOR NORMAL FEMALE WHITE MICE, 1921-23.

(Accidental deaths excluded.)

Age Period.	Per cent. of all animals dying within this period.	Per cent. of survivors dying within this period.	Per cent. of all animals dead at end of this period.
210–500 days	25·0	25·0	25·0
500–600 ,,	7·1	9·5	32·1

Age Period.	Per cent of all animals dying within this period.	Per cent. of survivors dying within this period.	Per cent. of all animals dead at end of this period.
600–700 „	25·0	36·8	57·1
700–800 „	21·5	50·0	78·6
800–900 „	17·8	83·5	96·4
900–1000 „	3·6	100·0	100·0

Mean duration of life, 652 ± 19 days.

TABLE 8.

MORTALITY STATISTICS FOR FEMALE WHITE MICE RECEIVING ACID OXIDATION PRODUCTS OF CHOLESTEROL, 1921-23.

(Accidental deaths excluded.)

Age Period.	Per cent. of all animals dying within this period.	Per cent. of survivors dying within this period.	Per cent. of all animals dead at end of this period.
210–500 days	16·0	16·0	16·0
500–600 „	16·2	19·2	32·2
600–700 „	19·6	28·6	51·8
700–800 „	25·8	53·3	77·6
800–900 „	12·8	57·2	90·4
900–1000 „	9·6	100·0	100·0

Mean duration of life, 678 ± 20 days.

A STUDY IN ORGAN SPECIFICITY

by

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The experiments recorded here were carried out as part of an investigation into the source of urinary protein in uranium nephritis in the cat. We were not concerned with the production or specificity of cytotoxins, but with the question as to whether it was possible by anaphylactic and complement fixation reactions to differentiate between protein from the blood, liver, and kidney. Reference to the more important observations in the literature is to be found in the papers by Fleisher and his colleagues (2), who have been able to show a relative organ specificity in the case of the liver and kidneys of guinea-pigs, by means of complement fixation in cross fixation and absorption experiments. The antigens and anti-sera appear from their observations to be complex in nature and to be composed of several partial antigens and immune bodies, one group of which has a relationship to all the tissues of the species. Precipitin and anaphylactic experiments seem to be less delicate. Pearce, Karsner, and Eisenbrey (3), using as antigens dog serum and albumins, globulins and nucleoproteins derived from the perfused liver and kidney of the dog, were unable to show any organ specificity in precipitin tests against the sera of immunized rabbits. Anaphylactic experiments made by the intravenous injection of guinea-pigs sensitized with these antigens showed that while animals sensitized with liver proteins reacted more actively to these than to kidney proteins, and those sensitized with kidney proteins were most affected by proteins from the same organ, all these sensitized animals reacted just as actively to serum. They did not attempt to make the reactions quantitative, for which purpose a very large series of animals would have been necessary. Salus (4) found that guinea-pigs sensitized with human serum did not react anaphylactically with blood-free kidney protein, and those sensitized with kidney protein were only slightly affected by the injection of serum. He obtained similar results with precipitin tests. The serum of rabbits immunized with human serum did not react with kidney protein, and conversely that of rabbits injected with kidney protein gave neither precipitation nor complement fixation when tested with blood serum.

Methods.

For the preparation of antigens cats were anaesthetized with ether, and their ventral surfaces shaved and prepared with spirit. The operation was carried out with full aseptic precautions. An incision was made in the mid-line from the xiphisternum to the pubic symphysis. The aorta was exposed by displacing the bowel out of the abdomen to the right, and the vessel was tied just above its bifurcation. A cannula was inserted above this ligature towards the heart, and the animal bled out as far as possible into a sterile vessel. The cannula was then removed and replaced by another provided with a bubble trap and connected with a tube leading from the bottom of a large reservoir of sterile saline placed about four feet above the operating table. The thorax was now opened, the aorta tied cranial to the diaphragm, and the inferior vena cava opened just before its entry into the auricle. The flow of saline was commenced without delay. The kidneys were completely decapsulated, the ureters cut, and a cannula was tied into the gall bladder after packing off carefully from the abdominal viscera with sterile swabs. In this way the kidneys were perfused through the renal arteries, and the liver through the portal vein as well as the hepatic artery. About 10 litres of saline were used for each perfusion, which took about two hours. The kidneys were now removed, and the cortex was chipped off with scissors into a weighed sterile petric dish. Pieces of liver were also removed, and placed in another similar vessel. The tissues were now ground up in sterile mortars, saline was added in proportion to their weight, and they were then placed in the ice chest overnight. The next day the supernatant fluid was pipitted off and cleared by centrifugation. The clear extracts were used without the addition of any antiseptic for immunization of rabbits and to test the sensitized uteri of virgin guinea-pigs. For use as antigen in complement fixation experiments carbolic acid was added to a strength of ·5%.

Rabbits weighing 1·5 kilos were immunized by the intravenous injection of 1 cc. of cat serum, and others by injecting 2 c.cm. of the extracts of liver and kidney on each of four successive days. These animals were anaesthetized and bled out aseptically from the carotid artery ten days after the last injection. Portions of the sera so obtained were absorbed for one hour at 37° C., using the fresh liver and kidney pulps from the preparation of extracts in the proportion of one part to four of serum, as in Fleisher's experiments. For this purpose the tissue pulps were washed four times with sterile saline. After absorption the sera were cleared by centrifugation, and by further absorption with sterile barium sulphate at room temperature we guarded against any anti-complemetary action acquired during the absorption. Incidentally we found, as Fleisher had, that this treatment did not alter the complement fixing power of the sera in the presence of various antigens.

The cross fixation experiments with rabbit sera were carried out, using a

haemolytic system 0·5 cc. in volume, made up as follows: Serum 0·1 cc., antigen 0·1 cc., saline 0·1 cc., complement in saline (3 M.H.D.) 0·1 cc., incubated for one hour at 37° C., after which 0·1 cc. of sensitized sheep red blood corpuscles (3% suspension) was added. The controls were made up as follows: (1) 0·1 cc. complement, 0·3 cc. saline, and 0·1 cc. sensitized red cells; (2) 0·1 cc. complement, 0·2 cc. saline, 0·1 cc. antigen, and 0·1 cc. sensitized red cells. The results of the whole test were read when the controls were completely haemolysed.

Before putting up the main experiment the sera and antigens were tested for anti-complementary action. For this purpose they were put up undiluted and in dilutions of 1 in 2, 1 in 4, 1 in 8, 1 in 16, and 1 in 32. All the sera were inactivated for twenty minutes at 56° C. before these tests were carried out.

In the main experiments the antigens were used in a fixed dilution determined by the previous investigation of their anti-complementary power. The sera were tested undiluted and in dilutions of 1 in 2, 1 in 5, 1 in 10, 1 in 20, 1 in 25, 1 in 30, 1 in 40, 1 in 50, 1 in 100, and 1 in 200.

For anaphylactic experiments virgin female guinea-pigs weighing about 180-200 gms. were sensitized by the injection of 0·5 cc. of cat serum subcutaneously, and others by the subcutaneous or intraperitoneal implantation of small pieces of freshly perfused liver and kidney. The plain muscle of these sensitized animals was tested usually about a month later, using the method of Dale and Laidlaw (1), in a bath of oxygenated Ringer of 120 cc. capacity. The sera of these sensitive animals was also used for complement fixation experiments. In these the sera were not absorbed, and were used undiluted and in dilutions of 1 in 3, 1 in 5, 1 in 10, 1 in 20, 1 in 30, and 1 in 40. These dilutions were tested against the antigens in dilutions of 1 in 3, 1 in 5, 1 in 10, and 1 in 20, which were not by themselves anti-complementary.

Cross Fixation and Absorption Experiments with Rabbit Sera.

The results of one of these experiments are recorded in Tables 1 and 2. In these P = undiluted serum, C = Controls, + indicates complete inhibition, ± almost complete inhibition, $\overline{+}$ inhibition of a less degree, and Tr that inhibition was barely evident. Table 1 shows the results obtained with the serum of rabbits immunized with liver, and with the same serum absorbed with liver and with kidney respectively, when tested against three antigens, cat serum, and kidney and liver extracts. Table 2 shows the corresponding set of results with the serum of rabbits immunized with kidney. The antigens were used in this particular experiment in the following dilutions, which were found to have no anti-complementary action: Cat serum 1 in 4, liver extract 1 in 5, and kidney extract 1 in 10. The original extracts were made by adding 61 c.cm. of saline to 28 gms. of ground-up liver pulp and 26 c.cm. of saline to 12 gms. of kidney cortex. It will be seen that our results for cat liver and kidney extracts confirm those obtained by Fleisher and his co-workers with guinea-pig tissues. Liver

immune serum (Table 1), while showing no cross fixation with cat serum, fixes complement at higher dilutions with kidney antigen than with liver, though specificity is shown by the complete removal of fixing power against liver antigen

TABLE I

	Antigens	Dilutions of Immune Serum P	1/2	1/5	1/10	1/20	1/25	1/30	1/40	1/50	1/100	1/200	C
Unabsorbed Serum	Cat Serum	−	−	−	−	−	−	−	−	−	−	−	−
	Kidney	+	+	+	+	+	+	±	±	−	−	−	−
	Liver	±	+	+	+	±	−	−	−	−	−	−	−
Serum absorbed with Liver	Cat Serum	−	−	−	−	−	−	−	−	−	−	−	−
	Kidney	+	+	+	−	−	−	−	−	−	−	−	−
	Liver	−	−	−	−	−	−	−	−	−	−	−	−
Serum absorbed with Kidney	Cat Serum	−	−	−	−	−	−	−	−	−	−	−	−
	Kidney	+	±	−	−	−	−	−	−	−	−	−	−
	Liver	+	+	+	±	−	−	−	−	−	−	−	−

TABLE II

	Antigens	Dilutions of Immune Serum P	1/2	1/5	1/10	1/20	1/25	1/30	1/40	1/50	1/100	1/200	C
Unabsorbed Serum	Cat Serum	−	−	−	−	−	−	−	−	−	−	−	−
	Kidney	+	+	+	+	+	+	+	+	+	+	±	−
	Liver	−	−	−	−	−	−	−	−	−	−	−	−
Serum absorbed with Kidney	Cat Serum	−	−	−	−	−	−	−	−	−	−	−	−
	Kidney	+	+	+	±	±	tr	−	−	−	−	−	−
	Liver	−	−	−	−	−	−	−	−	−	−	−	−
Serum absorbed with Liver	Cat Serum	−	−	−	−	−	−	−	−	−	−	−	−
	Kidney	+	+	+	+	+	+	+	+	+	±	−	−
	Liver	−	−	−	−	−	−	−	−	−	−	−	−

by absorption with liver pulp, while absorption with kidney pulp leave the titre against liver almost unchanged. On the other hand, kidney immune serum (Table 2) shows no fixation with liver antigen while fixing complement at high dilutions with kidney extract. Absorption with liver leaves this complement fixing power unchanged while absorption with kidney reduces it markedly. The proteins in kidney extract are therefore much more specific antigenically than are liver proteins.

It will be noted that in the reaction of the serum of a rabbit immunized with liver extract (Table 1) there is some indication of a "zone" phenomenon. This was still more evident when liver extract in a dilution of 1 in 2 was used as antigen, as this failed to fix complement with the undiluted immune serum, but reacted with dilutions of the serum from 1 in 2 to 1 in 40. The possibility of the occurrence of a "zone" in which no reaction occurs makes it important for the interpretation of negative reactions of immune sera with serum or urinary protein that these should be tested in a series of dilutions which are not by themselves anti-complementary.

With the sera of rabbits immunized with cat serum our results have so far been unsatisfactory, as they have failed to give complement fixation with serum except in the lower dilutions, and have given good fixation at somewhat higher dilutions with kidney extracts, and this even when there was a fair precipitin titre for cat serum. We have not yet been able to obtain sera with a high complement fixation titre for cat serum protein. In view of this the fact that immune organ serum gave no trace of reaction with cat serum in any dilutions which we tested must not necessarily be interpreted as indicating either that the organ extracts used for immunization were wholly free from serum protein or alternatively that serum and the liver and kidney extracts do not share a common antigen. The difficulty which we encountered in trying to produce complement fixing rabbit sera for cat serum is also interesting in the light of the findings in our complement fixation experiments with the serum of guinea-pigs sensitized with cat serum and killed about thirty days after the sensitizing injection. In these there was no fixation with either serum or kidney antigen in any dilution tested. On the other hand the serum from guinea-pigs sensitized with kidney gave good fixation when tested undiluted and in dilutions of 1 in 3, 1 in 5, and 1 in 10 against kidney antigen in a dilution of 1 in 3, and failed to react with cat serum in any dilution. The sera of other guinea-pigs sensitized with cat kidney also failed to react with liver extract. In guinea-pigs sensitized with cat liver the serum undiluted and in dilutions of 1 in 3 and 1 in 5 fixed complement with liver antigen in all the dilutions tested. They also gave some fixation with kidney antigen in a dilution of 1 in 3, though not with the higher dilutions of antigen.

Anaphylactic Experiments.

So far as we are aware, the isolated plain muscle preparation has not before been used in the investigation of this problem, though it presents obvious advantages over the method of injecting the whole animal. Since the uterine horns

are of equal sensitiveness, it is possible to test each with a different organ extract, and an additional advantage is offered by the possibility of testing for residual sensitiveness to other antigens after exhausting the reactions to reasonably large doses of one. We were careful in these experiments not to use doses large enough to have a direct stimulant action on normal plain muscle.

Experiments with Guinea-pigs sensitized by Implantation of Liver.

Several series of these were tested, and the most sensitive gave a maximal reaction with 0·05 cc. of liver extract, which by Kjeldahl contained 2·6% of protein, and which in a dose of 0·1 cc. gave no trace of response with the uterus from a guinea-pig sensitized with cat serum whose other horn reacted maximally to 0·01 cc. of serum containing 7·52% protein. Treatment of the plain muscle from a guinea-pig sensitized to serum, with this dose of liver extract only slightly diminished, the extent of its subsequent response to 0·01 cc. of serum, and afforded no evidence of the presence of serum protein in the extract.

The uteri of guinea-pigs of this series one month after sensitization with cat liver when tested with kidney extract containing 2·9% of protein reacted maximally to doses of 0·1 cc. and to 0·01 cc. of serum. When the sensitiveness of the plain muscle to liver protein was exhausted by repeated additions of this extract to the bath they still reacted to a dose of 0.1 cc. of serum, though not to half that amount. On the other hand, when the sensitive plain muscle was treated with serum in repeated doses till it no longer gave a response to 0·05 cc., it still gave a small though definite reaction with 0·05 cc. of liver extract. It is clear then that liver protein can be differentiated from serum protein by means of the anaphylactic reaction. The specificity may only be relative, or alternatively our results may be explained by the presence of some serum protein in the liver used for sensitization. The latter is the most probable explanation, since it seems not unlikely that in the course of saline perfusion of an organ, however thorough, there may in the early stages be some leakage of serum protein out of vessels damaged by the perfusion of a fluid of low osmotic pressure into the surrounding tissue cells. It is not possible to differentiate between liver and kidney protein by means of the anaphylactic reaction. When the sensitiveness of a horn is exhausted to liver protein it will no longer react to kidney extract (even in a dose of 0·5 cc.), nor alternatively will it react to liver after exhaustion with kidney extract. The possession of a common antigen which plays a dominant role in sensitization by liver and kidney extracts furnishes the easiest explanation of this result.

Figure 1 is a good illustration of the method used, and serves very well to exemplify some of the findings. It is the tracing obtained from the two horns of a guinea-pig of another series thirty days after sensitization by implantation of perfused liver. It will be noted that the plain muscle is more sensitive to

liver than to kidney extract, though the degree of sensitization is not so great as in the experiments described above.

The right horn was treated with successive doses of 0·1 cc., 0·5 cc., 0·5 cc., 1·0 cc., and 0·5 cc. of kidney extract. The bath was washed out after each response There was no response to the last dose of kidney extract, showing that

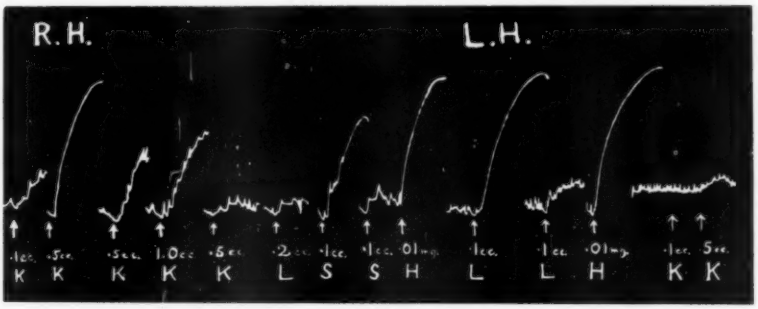

Fig. 1. Reactions of the isolated uterus of a guinea-pig sensitized by implantation of perfused cat liver. R.H. = right horn. L.H. = left horn. K. = kidney extract. L. = liver extract. S. = cat serum. H. = histamine.

desensitization had been achieved. A dose of 0·2 cc. of liver extract now caused no response though after changing the Ringer 0·1 cc. of cat serum gave a two-thirds maximal response, and suggested the presence of some blood serum in the tissue used for sensitization. Another similar dose of serum after changing the Ringer evoked no response. The response to 0·01 mgm. histamine shows the maximal contraction of the horn. The left horn gave a maximal response to 0·1 cc. of liver extract, and was then partially desensitized, giving only a very small response to a second dose of the extract after changing the Ringer. 0·01 mgm. of histamine added to the bath evoked a maximal response, and subsequent addition of 0·1 cc. and 0·5 cc. of kidney extract caused no appreciable effect.

The degree of desensitization in all these experiments is relative. For instance, the very small second response to 0·1 cc. of liver extract would doubtless have been a much larger one had 1·0 cc. of liver extract been used in its stead. The two doses were, however, sufficient to desensitize to the subsequent action of kidney extract in doses of 0·1 and 0·5 cc. It will be noted also that the response to 0·1 cc. of liver extract is much greater than that to 0·1 cc. of kidney extract, though these were of approximately equal strength, as far as content of protein was concerned. It should be remembered, however, that some part of the proteins present (the nucleoproteins) are not antigenically active.

Experiments with guinea-pigs sensitized by Implantation of Kidney Cortex.

These were much less regularly sensitive than guinea-pigs sensitized with liver, and in the series corresponding to our most sensitive series of guinea-pigs in which liver was implanted, the plain muscle reacted maximally at best with 0·1 cc. of a kidney extract which contained 2·9% of protein, and which failed to cause any reaction in this dosage in the plain muscle of a guinea-pig sensitized with cat serum and reacting maximally with 0.01 cc. of serum. They were likewise much less sensitive to serum protein, the uterus giving only a small reaction with 0·1 or 0·2 cc. of serum, but after thorough desensitization with serum protein sensitiveness was still unimpaired to doses of 0·1 and 0·2 cc. of kidney

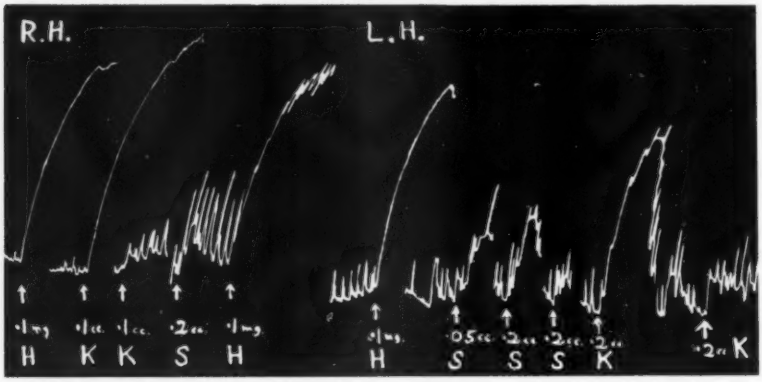

Fig. 2. Reactions of the isolated uterus of a guinea-pig sensitized by implantation of cortex of perfused cat kidney. R.H. = right horn. L.H. = left horn. K. = kidney extract. L. = liver extract. S. = cat serum. H. = histamine.

extract. Previous response to kidney protein did not appear to affect its sensitiveness to serum. Figure 2 is from an experiment in this series of guinea-pigs sensitized thirty days before by the implantation of kidney cortex which illustrates these points. The right horn has been tested with 0·1 mgm. of histamine, and gave a maximal response. Kidney extract in a dose of 0·1 cc. caused a similar response, but a second dose after changing the Ringer gave only a barely appreciable effect. A small response was then caused by 0·2 cc. of serum and 0·1 mgm. histamine, added without washing out the bath, gave a stepped but almost maximal response, probably because a large part of the dose became entangled in the protein froth. The left horn gave a maximal response to histamine, and then a series of responses to doses of 0·05, 0·2, and 0·2 cc. of serum, the last causing only a very small rise. The plain muscle was still sensitive to

kidney extract for a dose of 0·2 cc. gave a three-quarter maximal reaction. A second dose after changing the Ringer caused only a small response.

With liver extract containing 2·6% of protein the uteri of guinea-pigs of this series reacted in a dose of 0·1 cc., though after desensitization with sera and a response to 0·2 cc. of kidney extract, they gave no response to the same dose of liver extract. Figure 3 is taken from another set of experiments. The right horn has been treated with three successive doses of 1·0 cc. of liver extract, and is then shown to have lost its sensitiveness to kidney extract. The left horn responds to a dose of 0·1 cc. of serum with a small contraction, gives a maximal response to 2 c.cm. of kidney extract, and is thereafter desensitized not only to kidney but also to liver, as is shown by its failure to react to a dose of 1·0 cc. of liver extract. The anaphylactic plain muscle of guinea-pigs sensitized with either

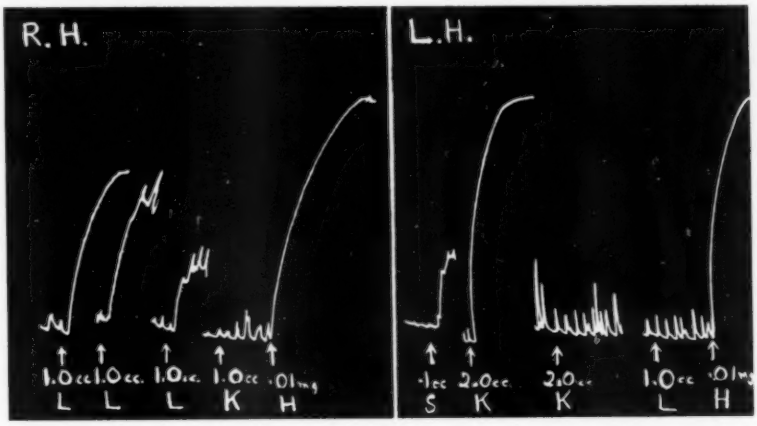

Fig. 3. Reactions of the isolated uterus of a guinea-pig sensitized by implantation of a cortex of perfused cat kidney. R.H. = right horn. L.H. = left horn. K. = kidney extract. L. = liver extract. S. = cat serum. H. = histamine.

liver or kidney appears to be unable to differentiate between extracts of these two organs and the attendant sensitiveness to serum, removal of which by desensitization does not greatly affect the response to kidney or liver extracts, is probably to be accounted for by incomplete removal of serum protein in the perfusion of tissues for implantation.

Experiments with Guinea-pigs sensitized with Cat Serum.

The plain muscle of these animals usually reacted maximally with a dose of 0·01 cc. or less of serum. As mentioned above, in one set of experiments there

was no reaction with 0·1 cc. of kidney and liver extracts and only a small decline of sensitiveness of the plain muscle following such treatment as estimated by the subsequent response to cat serum. In larger doses the tissue extracts often caused the sensitized plain muscle to respond, though even in this case repeated treatment with the tissue extracts did not desensitize the plain muscle to doses of cat serum of the order of 0·2 cc., which gave rise to maximal responses. We are inclined to attribute these occasional reactions to the presence of traces of serum protein in the extracts of the perfused organs, rather than to relative specificity as between serum and organ extracts.

CONCLUSIONS.

1. Extracts of the perfused liver and renal cortex of the cat can be distinguished by means of cross fixation and absorption experiments, though not by the anaphylactic reaction of the sensitive plain muscle of guinea-pigs.

2. This last method readily distinguishes both extracts from the serum of the cat.

3. A common antigen (or antigens) plays an important role in sensitization with these tissues.

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ON THE STIMULATING EFFECT OF PHOSPHATIC FERTILIZERS ON AZOFICATION IN SOUTH AUSTRALIAN SOILS

by

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In Europe, and a great part of North America, the maintenance of the nitrogen supply of the soil is a phase of soil fertility which has received much consideration, both from the scientist and the practical agriculturist. In these countries nitrogen, in many soils, is the limiting factor for crop production. The supply of soil nitrogen is almost everywhere inadequate for crop requirements, and it is possible to calculate approximately the time necessary for its exhaustion, provided no fresh supplies are meanwhile made available either by the application of nitrogenous fertilizers or by stimulation of the nitrogen-fixing micro-organisms of the soil.

A consideration of the farming practices current in South Australia—the "bare-fallow-wheat" or "bare-fallow-wheat-pasture (or barley)" rotations—with reference to this effect on soil fertility, at once brings us up against this problem of the nitrogen supply, but from a quite different aspect to that considered above. Owing their development and almost universal adoption to exigencies of either climate or economic circumstances, these practices necessitate a heavy and continuous drain on the nitrogen reserves of the soil. Nitrogen removed from the land in grain crops or stock is not returned to it under normal circumstances in a combined form, either directly or indirectly. The rapid translation of plant proteins to the grain as maturation proceeds almost completely depletes the straw and flag of the cereal crops of their nitrogenous material. The consumption of the bulk of this grain, and of meat also, in oversea countries, or in our own densely populated cities, leads to a considerable loss of combined nitrogen, since not infrequently the sewage with which it is incorporated is discharged into the ocean, where any decomposing nitrogenous material is rapidly subjected to denitrification processes.

In view of this continuous drain on the soil nitrogen reserves, the inability of nitrification to maintain a supply of available nitrogen compounds commensurate with the needs of each succeeding crop, is at once evident. The amount

of organic matter available for nitrification—apart from the exceptional cases where dressings of farmyard manure are applied—is restricted to the plant residues and animal droppings occurring on the particular land in question. Yet, notwithstanding that the vast majority of our South Australian soils are lower in their total nitrogen content than most of those of Europe or North America, it has been repeatedly demonstrated in our principal wheat-growing areas that nitrogenous fertilizers are not required to obtain maximum crops of either grain or hay. On the contrary, the rank sappy growth so frequently observed where some form of nitrogenous fertilizer has been applied, seems to indicate a superabundance of this essential element. Nitrogen from some source, sufficient at least to meet the needs of a cereal crop grown biennially on the same land, is therefore being made available continually, Since analyses show such inadequate amounts of total nitrogen in these soils, we are forced to the conclusion that the source of this essential element must be the atmosphere itself. Numerous species of soil bacteria are known to possess the capacity of assimilating atmospheric nitrogen in some degree. This may subsequently become available for the needs of plant life by nitrification of the decayed remains of these bacteria. However, in this connection we may disregard for the present the symbiotic nitrogen-fixing bacteria *Pseudomonas radicicola* (Beij), since leguminous crops in this State are relatively unimportant except in a few districts specially favoured either climatically or topographically. Of the various groups of free-living nitrogen-fixing bacteria known to occur in the soil the genus *Azotobacter* of Beijerinck has attracted the greatest attention of workers on account of its energetic fixation of nitrogen in culture media, and the evidence seems to be consistent with the view that the *Azotobacter* group is of chief importance in the soil. Records show that it is most widely distributed under tropical and semi-arid conditions, being frequently absent from the soils of the colder and wetter countries. Evidence accumulated by the writer[1] denotes the universal distribution of a vigorous *Azotobacter* flora in South Australian soils. Species of *Azotobacter* have been isolated from every one of a large number of soil samples collected in different parts of the State, ranging in type from a light calcareous sand carrying virgin mallee scrub, to heavy, black, alluvial truck soil. In every instance also a dense *Azotobacter* film was formed in Ashby mannite media (1) within six days at an incubation temperature of 30°C. Bearing in mind the almost indispensable rôle which phosphatic fertilizers have come to play in Australian agriculture, the possible stimulatory effect of this manurial practice on the extent and rate of azofication in local soils was made the basis of the investigation about to be described.

According to Stoklasa (5), *Azotobacter* uses a gram of phosphorus for every .5 to 5·7 grams of nitrogen assimilated. It would appear, therefore, that the functioning of these organisms is directly dependent upon a readily available

[1] Unpublished data from investigations not yet completed.

supply of phosphorus. An analysis of *Azotobacter* cells by Stoklasa adds further weight to this hypothesis. He found them to contain 10·2 per cent. of total nitrogen and 8·6 per cent of ash. The ash contained from 58 to 62·35 per cent. of phosphoric acid. Christensen (2) in Denmark has found soils in which he considers phosphorus to be the limiting element in *Azotobacter* growth. He concludes that where a mannitol solution free from phosphorus does not produce an appreciable growth of *Azotobacter* after inoculation with a soil, it may be assumed that the soil is deficient in available phosphorus.

The experimental method adopted in this investigation was suggested by Christensen's work. The amount of nitrogen fixed in a liquid media of known phosphate concentration, and containing a measured quantity of sterilized soil, was taken to be an index of the stimulating effect of added phosphates on the *Azotobacter* flora of such soil. Thus the phosphate content of the soil was made the variable factor, the organism used for the fixation of nitrogen being the same throughout.

EXPERIMENTAL.

Into 300 cc. Erlenmeyher flasks, each containing 10 grams of a finely-powdered, air-dried soil sample, were measured 100 cc. portions of a 2% nitrogen-free mannite solution of the following composition (6):

Mannite	20·0 grams
Magnesium sulphate	0·2 grams
Sodium chloride	0·2 grams
Calcium carbonate pptd.	5·0 grams
Distilled water	1,000 cc.

The reaction was adjusted neutral to phenolphthalein.

The media used for one-half the flasks was free from phosphates; the remainder contained added phosphorus as potassium di-phosphate in 0·02% concentration, i.e., 0·2 grm. per litre. The flasks were sterilized in the autoclave at 15 lbs. pressure for one and a half hours, and all except the controls inoculated with a twenty-four hour culture of *Azotobacter* on Ashby mannite agar. Nine flasks of each soil sample were prepared; three inoculated containing added phosphate, three inoculated without added phosphate, and three uninoculated for controls. After twenty-one days' incubation at 30°C. the amounts of nitrogen fixed were determined by Kjeldahl's method. The contents of the culture flasks were transferred to Kjeldahl digestion flasks by repeated rinsing with boiling distilled water acidified with sulphuric acid. The excess of water was carefully evaporated from the digestion flasks, and the estimation for total nitrogen then carried out in the usual way.

The six soils employed in this experiment are designated H, I, K, L, N, and O respectively. Soil samples H and I were collected from rich, heavily manured garden land at Clare. The remainder were obtained from the farm of the Roseworthy Agricultural College. These soils were selected for two reasons,

namely, (a) they are representative of widely varying types of cultivated soils in this State, from districts markedly different in their topography and climatic conditions; and (b) detailed records are available of the manurial treatment and cultivation received by them for a considerable number of years past (3, 4).

All of the soils selected were sampled early in May, 1924, the depth of sampling being six inches. A brief description of their chief characteristics and previous treatment is here appended.

Soil H. A heavy, black, close-textured, and deep alluvial soil. Pugs in wet weather; dries out rapidly, cracking into wide fissures. Extremely fertile. Irrigated with well water throughout the summer months. For past forty years has been employed continuously for market-gardening purposes. Heavily manured annually, both with farmyard manure and artificial fertilizers. Annual rainfall, 25 inches.

Soil I. A deep, rich, friable brown loam; semi-alluvial. Rich in humus. Heavy manurial treatment similar to Soil H. Cropped with permanent lucerne; irrigated. Annual rainfall, 25 inches.

Soil K. Hungry, grey, sandy loam; carrying virgin mallee scrub. A dense travertine crust lies from 1 to 1½ feet below the surface. Has never received any manurial treatment. Annual rainfall, 17 inches.

Soil L. Taken from sand dune; predominantly (80-90%) red sand. Cropped to wheat; crop just through at time of sampling. Received a dressing of 2 cwt. 36-38% superphosphate at seeding. Annual rainfall, 17 inches.

Soil N. Friable, red, sandy loam. Typical of much of the arable land of the mid-northern agricultural areas of this State. Cropped with wheat; crop just through at time of sampling. Annual rainfall, 17 inches.

Soil O. Taken off a stubble plot adjacent to that from which sample N was collected. Both these plots are under "wheat-pasture-bare-fallow" rotation, and receive a drilled dressing of 1 cwt. 36-38% superphosphate at the seeding of the wheat crop every third year.

A repetition of the experiment gave a very satisfactory concordance of results to those first obtained, and the figures recorded below are the mean of the several determinations.

The following are the results obtained:

Soil.	Total Nitrogen in in 10 grms. soil (Milligrams).	Nitrogen fixed (Milligrams). P_2O_5 in media.	No P_2O_5 media.
H	21·2	15·2	4·4
I	15·3	13·4	8·4
K.	9·5	9·3	3·2
L	4·9	11·3	7·6
N	6·7	14·2	9·6
O	6·5	14·9	2·6

Figure 1 is a graphical representation of the data tabulated above.

DISCUSSION OF RESULTS.

It would appear from these figures that, other conditions being favourable, the application of superphosphate to certain types of soils may have a highly stimulating effect on the nitrogen-fixing bacteria. The most remarkable feature disclosed by an examination of the table given above is the great differences in nitrogen fixed in the media containing no added phosphorus. In this media a much greater fixation of nitrogen has taken place in the case of the cultivated soils

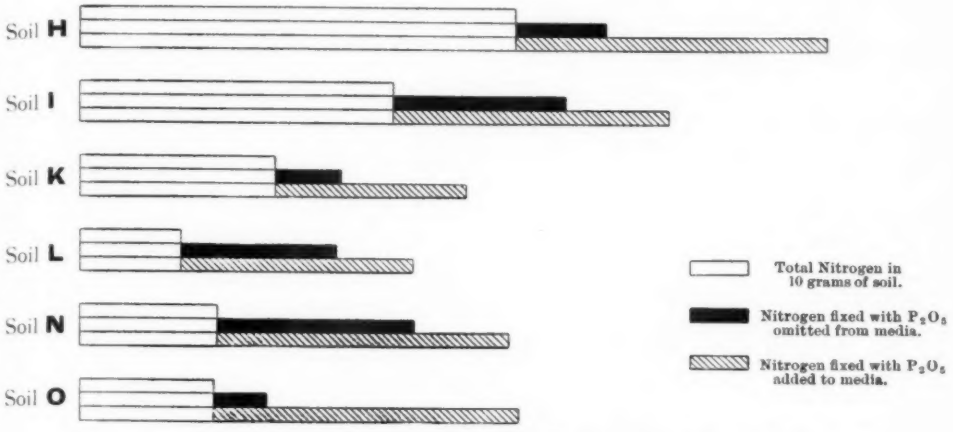

Fig. 1. Showing the effect of the addition of P_2O_5 on Nitrogen Fixation in some South Australian soils.

than with either the pasture soil O or the scrub soil K. Of these cultivated soils, L and N had received dressings of superphosphate at the rate of 2 cwt. and 1 cwt. per acre respectively, at seeding, two weeks prior to sampling; but no manure of any kind had been applied to either soil H or soil I for several months. These two last-named soils both have a high content of total nitrogen, which is possibly largely accounted for by the heavy dressings of farmyard manure applied to them annually for a number of years.

However, on the addition of excess of soluble phosphate to the media containing soils H and I, there was a pronounced increase in the amount of nitrogen fixed by *Azotobacter*. It is assumed in consequence that the available phosphate present in these soils is inadequate to maintain the *Azotobacter* flora at an optimum, measured by the amounts of the other constituents essential to their growth present. The stimulating effect of an application of a phosphatic fertilizer on the development of the nitrogen-fixing organisms of the soil is revealed by a consideration of the results obtained with the recently manured

soils L and N. The poor, extremely sandy soil L (2 cwt. super. at seeding), apparently almost devoid of humus, and of very low total nitrogen content, gave a nitrogen fixation in phosphate-free media amounting to 67% of that assimilated when excess of available phosphate was present. A previous experiment[2] gave evidence that this soil L contained a very vigorous *Azotobacter* flora at the time of sampling, notwithstanding its apparent barrenness. Strong support is thus given to the results recorded here indicating the stimulating effect of phosphatic fertilizers on azofication in Australian soils.

In the case of soil N (1 cwt. of super. at seeding) the nitrogen fixed in the phosphate-free media amounted to 68% of that fixed with phosphates present. Yet the soil O, collected from the pasture plot adjoining that from which sample N was taken, and hence of a strictly comparable type, gave very divergent results. Soil O, in phosphate-free media, gave a nitrogen fixation of only 10% of that obtained in the same media where soluble phosphate was present in excess. However, the pasture plot from which soil O was taken had not received any manurial treatment for two years. We are thus brought to the conclusion that the variation in amounts of nitrogen fixed bears a definite relationship to the amount of available phosphate present in the soil.

It will be noticed that the total nitrogen content of these two soils is practically the same. This is to be expected, since they are similar in character and cropped under the same rotation. Although the greater amount of nitrogen conceived to be fixed in the plot at present under cultivation does not appreciably increase the total nitrogen determination for that soil, this does not constitute an insuperable objection to the acceptance of the hypothesis. It is believed that the assimilated nitrogen incorporated in dead *Azotobacter* cells is made available to the crop throughout the period of growth by normal processes of decay and nitrification.

Another point raised by the table is the higher total nitrogen content of the virgin mallee soil K, and the greater relative fixation obtained in phosphorus-free media with this soil than with the soil O. Its higher total nitrogen content may be due partly to the continued accumulation of organic residues in the surface layers, and partly to the smaller demands made on its nitrogen reserves by sub-aerial vegetation. The higher relative nitrogen fixation with phosphorus-free media, about 35% of that obtained with excess of phosphates, as against 10% in the case of soil O, is more difficult to account for. One must conclude that the amount of available phosphorus in this soil is greater than in soil O, i.e., its original phosphate reserves have not been depleted by the production of annual cereal crops. In this connection it is interesting to note that heavy crops have frequently been grown without the aid of superphosphate on virgin land newly brought under cultivation.

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[2] From unpublished data.

SUMMARY.

An investigation into the effect of phosphates on nitrogen fixation by *Azotobacter* in some South Australian soil types is described, and the results tabulated and discussed. The conclusion is arrived at that the addition to such soils of soluble phosphate compounds, such as are contained in the artificial phosphatic fertilizers so extensively applied, may result in a pronounced stimulation of the natural azofication processes.

It is suggested, furthermore, that lack of available phosphoric acid is a limiting factor for *Azotobacter* growth in many South Australian soils, and the increased nitrogen fixation brought about by the application of phosphatic fertilizers may explain the general ineffectiveness of nitrogenous fertilizers in local agricultural practices.

It is proposed to continue and extend this work, and to investigate also the problem of a supply in these soils of readily assimilable carbohydrates essential for the energy transformations brought about by *Azotobacter* in the fixation of atmospheric nitrogen.

In conclusion, I wish to thank Professor J. B. Cleland for much helpful advice and kindly criticisms so freely given during the course of these investigations.

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